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TITLE: Deconstruction of Oncogenic K-RAS Signaling Reveals Focal Adhesion Kinase as a Novel Therapeutic Target in NSCLC

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14. ABSTRACT
About 25% of lung adenocarcinomas express mutant KRAS often is association with inactivation of the CDKN2A locus, which comprises $p16^{INK4A}$ and $p14^{ARF}$ or the p53 tumor suppressors. These mutations are associated with disease progression and poor prognosis. There are no therapies that target cancers that express mutant KRAS. We identified a RHOA-Focal adhesion kinase (FAK) axis that is required for the viability of mutant KRAS lung adenocarcinomas that carry INK4A/ARF mutations. This set of mutations is relevant since we determined that it occurs in about half of mutant KRAS lung cancers. We found that pharmacological inhibition of FAK caused tumor regression specifically in high-grade mutant Kras;$p16^{Ink4a}$/$p19^{Arf}$ null lung cancers in genetically engineered mice and in a large panel of cultured human lung cancer cells. We also found that mutant KRAS;p53 deficient lung cancers are vulnerable to inhibition of FAK, even though to a lesser extent. These findings provided the rationale for the implementation of a multi-institutional Phase II clinical trial using VS-6063 in lung cancer patients with KRAS mutations (PIs Dr. Gerber at UT Southwestern Medical Center). Ongoing work aims at the determining the underpinnings of the dependency of lung cancer cells to FAK both in cultured lung cancer cells and in vivo. In this regards, we found that FAK inhibition impairs the DNA damage response, potentiating the effects of radiotherapy.

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1. Introduction:

This application rests on the hypothesis that the genotype of cancer cells determines specific vulnerabilities that can be exploited in cancer therapy. The KRAS proto-oncogene is small guanosine triphosphatase that is constitutively activated (oncogenic KRAS) in 25% of non-small cell lung cancers (NSCLC). At present, there are no drugs that directly target oncogenic KRAS. With preliminary experiments we identified focal adhesion kinase (FAK) as a an essential requirement for the survival of NSCLC cells that express oncogenic KRAS and are deficient for either for the INK4A/ARF or p53 tumor suppressors. We proposed to test the hypothesis that FAK is a critical druggable vulnerability in NSCLC expressing oncogenic KRAS using an approach that integrates the use of lung cancer cell lines, engineered mouse models of mutant RAS lung cancer, RNA interference and targeted FAK inhibitors. We have made significant progress as demonstrated by the fact that our work provided the rationale for a Phase II multicenter clinical trial using defactinib a FAK small molecule inhibitor, to treat KRAS-mutated NSCLC patients (ClinicalTrials.gov Identifier: NCT01951690). This award does not directly fund this trial. This clinical trial has enrolled to date about 40 patients. With work performed during the past 12 months we have discovered that FAK inhibition leads to radiosensitizing effects, which we are characterizing with the goal of designing a future clinical trial to test the effect to radiotherapy in conjunction to FAK inhibition. Ongoing studies are addressing the mechanism that mediates the lung cancer cell death upon FAK inhibition, strategies to maximize the therapeutic effect of FAK inhibition and identify combination therapies that may synergize with dual PI3K/mTOR inhibition. This works takes advantage of cellular and mouse models of lung cancer.

2. Keywords

KRAS, lung cancer, mouse lung cancer models, FAK, targeted kinase inhibitors, RNAi, preclinical studies, radiotherapy, radiosensitization.

3. Accomplishments for grant W81XWH-12-1-0210.

3.1 What were the major goals of the project?

Goal 1. Determination of the anti-cancer effects of FAK inhibition in NSCLC cells. We propose to use genetic and pharmacologic approaches to establish whether FAK is required for the survival of NSCLC expressing oncogenic KRAS and deficient for either INK4a/ARF or for p53. We propose to: 1. Characterize the anti-tumor effects of FAK inhibition in NSCLC; 2 Determine the effect of genetic inactivation of FAK in oncogenic KRAS induced NSCLC tumorigenesis using a conditional knock-out allele of FAK.

Goal 2. Identification of strategies that synergize with inhibition of FAK to induce the death of NSCLC expressing oncogenic KRAS. Here we will use a genetic and pharmacologic approach in NSCLC cells and in mouse lung models to identify strategies to maximize cancer cell death upon pharmacologic inhibition of FAK. We propose to: 1. Determine whether FAK inhibition synergizes with inhibition of PI3K/mTOR signaling or of other druggable oncogenic signaling pathways in NSCLC cells; 2. Complete the validation of a whole genome siRNA screening to identify oncogenic KRAS synthetic lethal interactions in NSCLC cells.

For the purpose of this progress report we will follow the format mandated by the "technical reporting requirements" applied to the approved statement of work.

3.1 What was accomplished under these goals?

Goal #1. Determination of the anti-cancer effects of FAK inhibition in NSCLC cells.

Our preliminary data suggest that FAK is required for the viability of NSCLC cells expressing mutant KRAS and deficient for either INK4A/ARF or p53 (1). We propose to: 1. establish whether NSCLC cells are dependent on FAK; 2. Identify the mechanisms responsible for this dependency; 3. Identify additional mutant KRAS vulnerabilities.

Our preclinical data led to a phase II clinical trial with the FAK inhibitor VS-6063 (ClinicalTrials.gov Identifier: NCT01951690) in mutant KRAS lung adenocarcinoma patients. This trial is not directly funded by this grant and has enrolled about 40 patients to date. Our goal is to provide the framework for the development of FAK inhibitors in NSCLC.

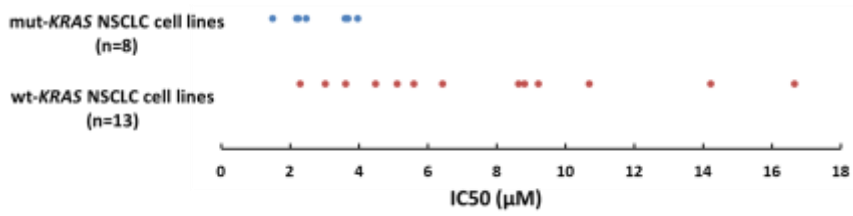


Fig. 1. IC50 distribution of PF-562271 in 21 lung cancer cell lines 72 hours after drug treatment. We used the following mutant KRAS cell lines: A549, H157, H460, H1264, HCC4017 (mutant KRAS;INK4a/ARF mutant) and H157,, H358, H1264, H1792, H2009, HCC4017 (mutant KRAS;p53 mutant). We used the following wild type KRAS cell lies: H125, H522, H596, H661, H920, H1650, H1975, H1993, H2073, H2228, H3122, HCC95, HCC827.

Subtask 1a. Pharmacologic inhibition of FAK in human NSCLC cells in vitro.

Major activity: As proposed in the statement of work, we tested the sensitivity of a panel of lung cancer cells (21 cell lines), which include the major mutations that occur in lung cancer, to FAK inhibitor PF-562271 (now renamed VS-6063) (2). We also tested the FAK inhibitor VS-4718, previously known as PND- 1186 (3).

Specific Objectives: the purpose of these studies is the determination of the sensitivity of mutant KRAS lung cancer cells to pharmacologic inhibitors of FAK.

Significant results: In viability assays, we found that PF-562271 inhibits every mutant KRAS cell line we tested with a half maximal inhibitory concentration (IC50) between 1 and 4 millimolar after 48 hours of incubation. This concentration is readily achievable in vivo both in mouse cancer models and in patients (2). In contrast, the IC50 for lung cancer cell lines encoding wild type KRAS ranged between 2 and 18 millimolar (Fig. 1). We obtained equivalent results with FAK inhibitor VS-4718 (data not shown).

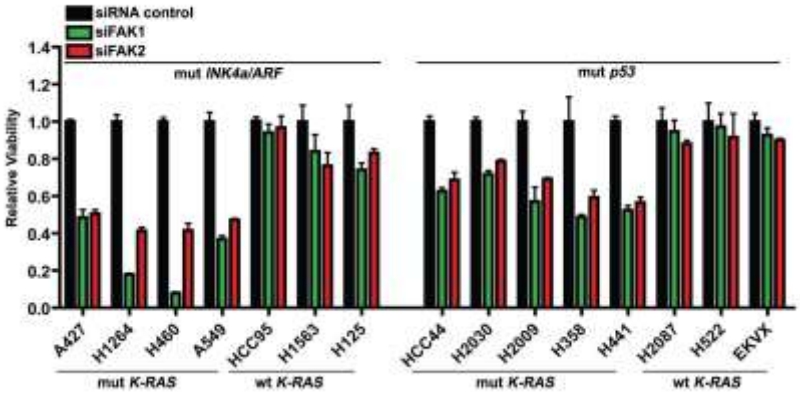


Fig. 2. FAK is a crucial vulnerability of mutant KRAS;INK4a/ARF - deficient NSCLC cells. The histogram shows viability of NSCLC cell lines 72 hours after transfection with siRNAs against FAK. The mutation status of the cell lines is indicated.

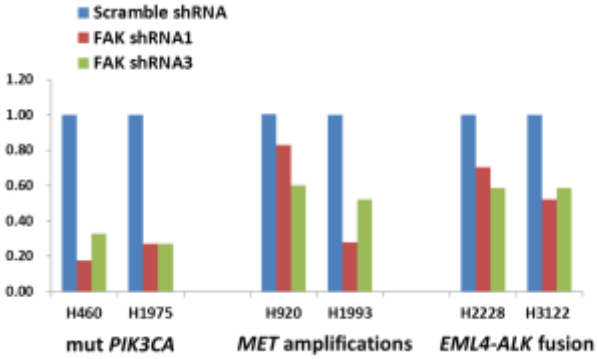


Fig. 3. FAK silencing in representative mutant PI3K (mut PI3K), c-MET amplified and EML4-ALK positive lung cancer cells. Note that FAK silencing decreases significantly viability in mutant PI3K lung cancer cells.

Last year we reported that in the same cell lines, FAK silencing resulted in al least 60% decrease in viability 48 hours after transfection in mutant KRAS;INK4A/ARF mutant cells and by about 50% in mutant KRAS;p53 mutant cells. There was less than 20% inhibition of viability in NSCLC cells that carry wild type KRAS and are mutant for either INK4a/ARF or p53 (Fig. 2).

Even though the inhibitory experiments with siRNA and pharmacologic inhibitors of FAK are qualitatively different and difficult to compare to each other, we speculate that pharmacologic inhibition of FAK is less selective than silencing. This discrepancy could be caused by non-specific off target effects of the pharmacologic inhibitors. Nevertheless, we conclude that pharmacologic inhibitors of FAK cause selective death of mutant KRAS lung cancer cells in vitro.

We also tested the ability of FAK silencing to negatively affect cellular proliferation of lung cancer cells carrying mutations of other dominant oncogenes. We found that FAK silencing exerts negative effect on cell viability in lung cancer carrying PI3K activating mutations, but not c-MET or EML4-ALK fusions (Fig. 3 shows representative examples). We conclude that suppression of FAK may be effective in PI3K mutant lung cancer.

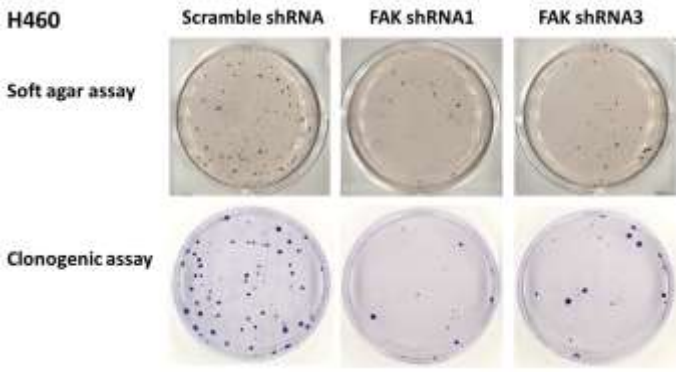


Fig. 4. Soft agar and clonogenic assay of H460 cells (mutant KRAS) after FAK silencing.

Cancer cells are endowed with the ability to grow in an anchorage-independent manner. For this reason we tested whether FAK silencing affect the ability of NSCLC cells to form colonies in soft agar. Indeed, we found that FAK silencing significantly reduces the ability of mutant KRAS lung cancer cells to grow in soft agar. This reduction was comparable to the reduction in clonogenic ability caused by FAK silencing (Fig. 4).

Since FAK has been implicated in the regulation of cell adhesion, we also tested whether FAK inhibition is detrimental to lung cancer cells cultures on fibronectin or collagen-coated plates, however we determined that these culture conditions do not impact sensitivity to FAK inhibition of silencing (data not shown).

We initially proposed to test the effects of the FAK inhibitor GSK-2256098 made by GlaxoSmithKline. However, GlaxoSmithKline has not published this compound yet. For this reason we have prioritized our studies on PF-562271 and VS-4718, which are published compounds. In addition, given the working relationship with Verastem, Inc. (the sponsor our the VS-6063 clinical trial), we reason that any compelling findings could be rapidly translated to the clinic.

In aggregate these experiments lead to the conclusion that pharmacologic inhibition of FAK lead to significant cytopathic effects in mutant KRAS lung cancer cells deficient for INK4a/ARF or p53 and also mutant PI3K. We also conclude that FAK promotes cell viability and proliferation independently of the engagement to the extracellular matrix.

Other achievements: NA

What do you plan to do during the next reporting period to accomplish the goals? We consider this subtask completed.

Subtask 1b: Determination of the functional consequences of FAK inhibition in NSCLC cells.

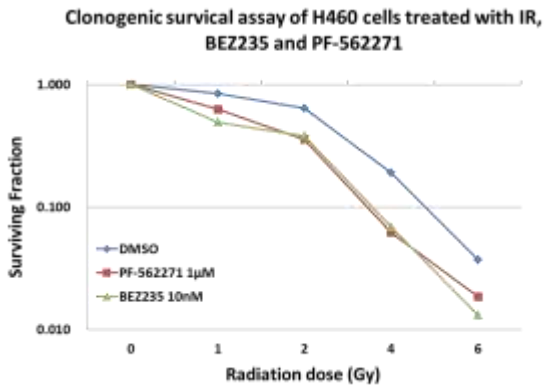


Fig. 5. FAK blockade sensitizes H460 cells to the effects of Ionizing radiations. The graph illustrates a representative clonogenic survival assays of H460 cells treated as indicated. Colony number was calculated from three replicate plates of three independent experiments. We used cells treated with the dual PI3K/mTOR inhibitor BEZ235, which is potently radiosensitizing, as a positive control.

Major activity: as proposed in the statement of work, we performed experiments in cultured lung cancer cells.
Specific Objectives: The goal of subtask 1b is to identify the cellular networks that mediate the antitumor effects of FAK suppression in mutant KRAS target gene in NSCLC.
Significant results: With experiments performed during the past year we determined that, surprisingly kin lung cancer cells, FAK inhibition does not affect significantly signaling pathways that in other cellular systems depend on FAK such as AKT, ERK or JNK. Since the procedures that we proposed in our initial proposal did not bear fruits, we used reverse phase protein array assays to determine the signaling networks affected by FAK silencing in H460, A549 and H4017 mutant KRAS lung cancer cells, which are representative of the panel of cells used in subtask 1a.

Surprisingly, this analysis revealed that FAK silencing downregulates several gene products implicated in DNA double strand break repair (Rad50, Rad51 and XRCC1, which have been implicated in non-homologous end joining). Accordingly, we found that pharmacologic inhibition of FAK with PF-562271 or with RNA interference (RNAi) leads to persistence of DNA damage foci and radiosensitization to an extent similar to the dual PI3K/mTOR inhibitor BEZ235, which we previously shown it is highly radiosensitizing (Fig 5 and data not shown) (4).

In last year's progress report we mentioned preliminary evidence showing that FAK physically interacts with the SUMO E3 ligase Protein inhibitor of activated STAT1 (PIAS1). This finding is coherent with the

observation of others that: 1. A fraction of FAK is SUMOylated and interacts with PIAS1 in the nucleus (5); 2. PIAS1 positively regulates the DNA double stand break repair response (6).

During this year of funding we have determined that: 1. *PIAS1* and *FAK* genes are co amplified in NSCLC cell lines (Fig. 6A); 2. FAK and PIAS1 proteins are noticeably elevated in metastatic lung tumors (Fig. 6B); 3. PIAS1 and FAK colocalize outside the cell nucleus prior to FAK nuclear entry (Fig. 6C); 4. FAK knockdown leads to significant reduction of PIAS1 protein (Fig. 6D).

Other achievements: Last year we published that two-hit inactivation of the INK4A/ARF tumor suppressor occurs in about 50% of mutant KRAS lung cancers. Furthermore, 20% of lung cancer also carries two-hit inactivation of the INK4B tumor suppressor, which is part of the CDKN2AB locus, which comprises also INK4a/ARF. We demonstrated that loss of INK4A results in rapid progression of mutant KRAS lung tumors to high-grade neoplasms (1). Loss of INK4B further hastens tumor progression (7). Notably, the mutant KRAS H460 and A549 lung cancer cell lines, which carry an homozygous deletion of INK4A/INK4B are sensitive to FAK inhibition (1, 7).

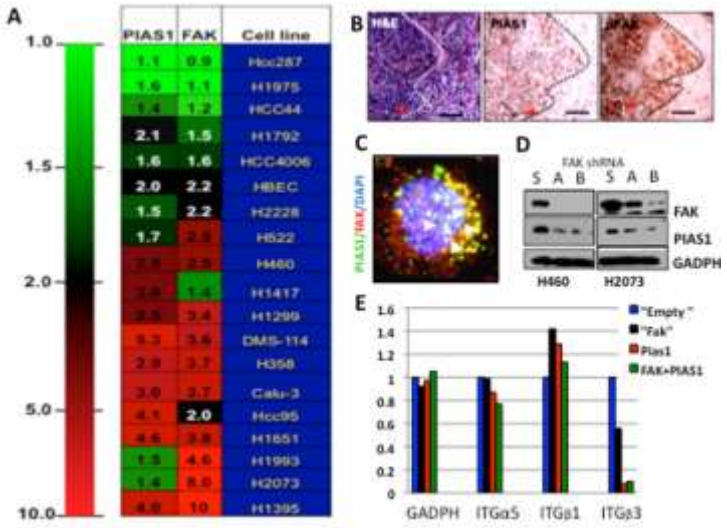


Figure 6. FAK and PIAS1 interact in lung cancer. A. The heatmap shows single nucleotide polymorphism (SNPs) copy number analysis of *FAK and PIAS1* genes in the indicated lung cancer cell lines. Note the concordant change in copy number for both genes. (**B**) Immunohistochemistry of Lung cancer metastasis to lymph nodes confirms that FAK and PIAS1 proteins are present in invasive lung cancer cells but not in the surrounding tissue. (**C**) Immunofluorescence shows perinuclear localization of FAK and PIAS1 proteins. (**D**) Western blot shows that FAK knockdown reduces PIAS1 protein levels significantly.

We also isolated clones of mutant KRAS lung cancer cells that developed acquired resistance to FAK silencing. These cells, despite adequate siRNA silencing, regain their proliferation and survival capabilities. We also intend to use these cells to identify the signaling networks that bypass the cell death caused by FAK knockdown. We reason that these experiments may identify mechanisms that mediate resistance to pharmacologic inhibition of FAK.

What do you plan to do during the next reporting period to accomplish the goals? We are currently validating the findings described above and addressing their mechanistic underpinnings. We will also functionally determine with xenograft experiments whether pharmacologic inhibition of FAK in combination with radiation therapy is an effective treatment strategy in mouse lung cancer models (see also subtask 1c).

This observation could lead to clinical studies since radiotherapy is a standard of care modality in the treatment of lung cancer.

Time frame: year 3.

Subtask 1c: Pharmacologic inhibition of FAK in human NSCLC cells in vivo.

Major activity: Treatment with pharmacologic inhibitors of FAK mice carrying xenografts of lung cancer cell lines.

Specific Objectives: Testing the antitumor effect of pharmacologic inhibition of FAK in human lung cancer cells grown as xenografts in mice.

Significant results: The experiments described in subtask 1a and 1b provide the framework for subtask 1c. Now that subtask 1a is completed, we are started testing the effect of pharmacologic inhibition of FAK in xenografts of human NSCLC cell lines in immunocompromised mice.

Other achievements: Our results regarding the radiosensitizing properties of FAK inhibition also provide the rationale for preclinical trials testing radiation therapy and FAK inhibition in immunocompromised mice carrying xenografts of human NSCLC cells.

What do you plan to do during the next reporting period to accomplish the goals? These experiments are in progress and will be completed during the last year of support.

Time frame: year 3.

Subtask 1d. Determination of anti-tumor effects due to pharmacologic inhibition of FAK in transgenic mice with NSCLC induced by oncogenic KRAS.

Major activity: Treatment with pharmacologic inhibitors of FAK genetically engineered mice with lung cancer induced by oncogenenic KRAS.
Specific Objectives: Testing the antitumor effect of pharmacologic inhibition of FAK in vivo in mouse models of lung cancer.
Significant results: Since the submission of the grant, we have determined that FAK inhibition causes striking inhibition of lung adenocarcinomas but not of lung adenomas of mutant *KRAS/Ink4a/Arf*$^{-/-}$ mice (1). Thus, it is of interest to determine whether treatment with FAK inhibition increases overall survival.

What do you plan to do during the next reporting period to accomplish the goals? For this purpose, we are considering testing the effects of pharmacologic inhibition of FAK in LsL-*Kras*, LsL-*Kras/p53*$^{-/-}$ and LsL-*Kras/Ink4a/Arf*$^{-/-}$ mice (8). Similar experiments with mice carrying xenografts are described in 1c. Should the experiments described in subtask 1c give a clear-cut answer, the experiments described in subtask 1d may be redundant and not informative. According we will execute these experiments after the completion of subtask 1c.
Other achievements: NA

Timeframe: year 3.

Subtask 1e. Determination of the impact of FAK deficiency in transgenic mice with NSCLC induced by oncogenic KRAS.

Major activity: Study the impact of Fak deficiency on mutant Kras lung cancer in genetically engineered mice.
Specific Objectives: determination of the impact of Fak deficiency on mutant Kras tumorigenesis in vivo in genetically engineered mice.
Significant results: We proposed to test the requirement of Fak for the progression from lung adenoma to adenocarcinoma. For this purpose, we are using mice carrying a floxed allele of *Fak* to generate cohorts of LsL-*Kras*/*Ink4a/Arf*$^{-/-}$ and LsL-*Kras/Ink4a/Arf*$^{-/-}$*/Fak*$^{f/f}$ mice (9). We have recently developed conditions to deliver the cre recombinase to the respiratory epithelium with lentiviruses that allow the generation of primary tumors and also of their metastatic spread. Thus, we will study the effect of FAK deficiency in primary and metastatic tumors.
Other achievements: Should these experiments demonstrate that Fak is required for lung tumorigenesis, we will perform experiments with mice carrying a knocked-in allele encoding an inducible Fak kinase dead mutant. This experiment will mimic genetically the action of a pharmacologic inhibitor of Fak and will discriminate whether the Fak kinase activity or its scaffolding function is important for tumorigenesis.

What do you plan to do during the next reporting period to accomplish the goals? These experiments are in progress and should mature during year 3.
Timeframe: year 3.

Goal #2. Identification of strategies that synergize with inhibition of FAK to induce the death of NSCLC expressing oncogenic KRAS.

We proposed to use genetic and pharmacologic approaches to identify strategies to optimize the therapeutic outcome of inhibition of FAK in NSCLC cells expressing oncogenic KRAS and either mutant INK4a/ARF or mutant p53.

Subtask 2a. Identification of combination therapies that synergize with pharmacologic inhibition of FAK;
Major activity: testing the effects of drug combinations in cultured lung cancer cells.
Specific Objectives: identification of combination therapies that synergize with inhibition of FAK.
Significant results: We prioritized our efforts to determine whether FAK inhibitors synergize with inhibitors of proteins that interact with FAK, such as c-MET, EGFR, SRC, PI3K, MEK1/2 or mTORC1/2. These experiments are not completed, but so far we have not identified synergistic relationships. This finding may indicate that in NSCLC cells, FAK signals though non-canonical pathways. This hypothesis is consistent with the observation that FAK silencing/inhibition downregulates components of the double strand repair machinery (refer to subtask 1a). If proven true this finding would reveal a novel and unexpected function of FAK, which could be harnessed for therapeutic purposes.

Other achievements: NA

What do you plan to do during the next reporting period to accomplish the goals? These experiments are in progress and we expect that these experiments will be completed by the end of year 3.

Time frame: year 3.

Subtask 2b. Complete the validation of a whole genome siRNA screening to identify oncogenic KRAS synthetic lethal interactions in NSCLC cells.
Major activity: Perform experiments to identify genes that when inactivated cause the death of mutant KRAS lung cancer cells .
Specific Objectives: Identification of additional mutant KRAS synthetic lethal interaction that could represent therapeutic targets.
Significant results: We completed the validation of the whole-genome synthetic lethal siRNA screening aimed at identifying genes that when are silenced induce cell death in mutant KRAS cells treated with the PI3K/mTOR inhibitor BEZ235 used at a 200 nM concentration (10). For the primary screening we used HCC44 NSCLC cells (mutant KRAS; mutant p53). At this concentration BEZ235 causes more than 80% inhibition of mTORC1/2 signaling, but less than 20% inhibition of cell viability. In the primary screening we compared the viability HCC44 cells treated with a control siRNA and 200nM BEZ235 to the viability of cells treated with siRNAs targeting individual tested genes treated with BEZ235. In parallel, we

	H2087	H460	HCC44	H23	H1819	A549
THBS3	y	y	y	y	y	n
HNRNPH2	n	y	y	y	n	y
GPX4	n	n	y	n	n	n
RRAGD	n	y	y	y	n	y
NDRG1	n	n	y	n	n	n
G22P	n	y	y	n	n	y
TBK1	n	y	y	y	n	y
WNK1	y	y	y	y	n	y
TBC1D14	NT	NT	y	NT	y	y

Table 1. The table shows the results of the secondary screening to identify genes that when silenced co-operate with the PI3K/mTORC1/2 inhibitor BEZ235 in causing death of lung cancer cells. Cell lines are indicated on the horizontal line. Genes on vertical line. Y (i.e. yes): cooperation between silencing and BEZ235 treatment; N (i.e no): no co-operation was observed. NT; Not tested.

determined the effects of silencing the targeted genes in cells treated with DMSO (the vehicle of BEZ235).

We considered positive hits genes that when silenced would cause less that 20% inhibition of viability but that would cause more than 70% viability in association with BEZ235. We chose to validate 50 of the top 300 statistically significant hits in HCC44. We chose genes based on the biological activity and availability for reagents to study their function. We confirmed 9 hits. Next, we determined whether their effects were generalizable to the following cell lines: HCC44, H460, H23 and A549 NSCLC cells as representative examples of mutant KRAS NSCLC cells and in H2087 (wild type KRAS;p53 mutant) an H1819 (no mutations noted in major oncogenes/tumor suppressor genes). Table 1 shows the genes that we have validated. RRAGD (Ras-Related GTP Binding D) is a GTP-binding protein implicated in the regulation of mTORC1. This finding suggests that a more complete inhibition of mTORC may have increased cytotoxic effects. TBK1 (TANK-binding kinase 1) has been implicated in transducing pro-survival signals in mutant KRAS lung cancer (11, 12). Notably, TBK1 inhibitors exist (BX795 and Amlexanox)(11, 13). Thus, we selected TBK1 for further functional studies in tissue culture.

The execution and validation of the synthetic lethal screening has been a very time consuming and labor intensive task. We learned that most synthetic lethal interactions occur only to the cell line used for the primary screening. Thus, there is a tremendous attrition of the "hit" that are generalizable across several cell lines. In addition, most of the hits found are not druggable or do not belong to networks that are druggable. Finally, there are issues of reproducibility of RNAi screenings across different platforms. These intrinsic limitations of the RNAi screening were recognized by NCI with the recently released SolveRAS RFA. This RFA invites projects for innovative and improved synthetic lethal screening. For this reason, we feel satisfied that our screening revealed FAK as a bona fide preclinical target. Accordingly, we consider the task of the validation of the synthetic lethal hits completed.

Other achievements: NA

What do you plan to do during the next reporting period to accomplish the goals? In case we detect synergistic effects using PI3K/mTOR inhibitors and TBK1 inhibitors, we will proceed with preclinical studies in lung cancer xenografts.

Time frame: year 3.

3.2 What opportunities for training and professional development has the project provided?

This project was not intended to provide training and professional development opportunities.
Dr. Mahesh Padanad (post-doctoral fellow), Dr. Ke-Jing Tang (Associate Professor at Sun Yat-sen University, Guangzhou, China, Visiting professor) and Niranjan Venkateswaran, MS (Research technician) conducted the research described in this progress report. Their involvement ij this project is part of their research training under the mentorship of Dr. Scaglioni, MD

3.3 How were the results disseminated to communities of interest?

I presented our data to the community meeting "*Conversations About Cancer" organized by* the American Cancer Society at the Collins Center of Southern Methodist University, University Park, Texas on 4/29/2014.

4. Impact.

With this research we have identified FAK as a novel targetable vulnerability of mutant KRAS lung cancer. Our preclinical data provided the rationale for the design and execution of a Phase II multi-institutional clinical trial in lung cancer patients which has enrolled about 40 lung cancer patients so far.

4.1. What was the impact on the development of the principal discipline(s) of the project.

Our research project indicates that mutant KRAS lung cancers that are also mutant for INK4A/ARF or for p53 are vulnerable to FAK silencing or pharmacologic inhibition both in cultured cells and in transgenic mice of high-grade lung adenocarcinoma.

This oncogenotype is of clinical relevance. We have determined that mutant KRAS;INK4A/ARF deficiency occurs in about 50% of mutant KRAS lung cancers. Mutant KRAS is present in 25% of the 175,000 newly diagnosed lung cancers that are diagnosed yearly in the USA yearly. Thus, the mutant KRAS;INK4A/ARF deficient genotype is present in about 20,000 to 25,000 patients/year in the USA. Mutant KRAS;p53 deficiency has a similar prevalence (7, 14).

We also identified a novel function of FAK, namely its ability to promote the repair of DNA damage. Our preliminary data indicate that FAK silencing or pharmacologic inhibition lead to defective DNA double-strand break repair causing significant radiosensitizing effects. Accordingly, FAK inhibition/silencing overcomes radioresistance of mutant KRAS lung cancer cells cultured in vitro. We are currently pursuing the hypothesis that FAK exerts its effect on the DNA damage repair machinery through the SUMO E3 ligase PIAS1.

Our data provided the rationale for the execution of the first clinical trial of a small molecule inhibitor of FAK in lung cancer patients (ClinicalTrials.gov Identifier: NCT01951690). This is a multicenter Phase 2 trial of the FAK inhibitor defactinib (VS-6063) to treat KRAS-mutated non-small cell lung cancer (NSCLC). This trial is actively enrolling patients (so far about 40). Treatment outcomes will be correlated to the presence of INK4A/ARF or p53 mutations.

4.2. What was the impact on other disciplines?

Our data indicate that the mutant KRAS cancer, which was considered a homogenous disease, can be sub-classified based on concomitant mutations and that specific oncogenotypes may dictate response to targeted therapy. It is conceivable that this paradigm may apply to cancer of other histological origins.

4.3. What was the impact on technology transfer?

A phase II multicentrer clinical trial sponsored by Verastem is ongoing (ClinicalTrials.gov Identifier: NCT01951690).

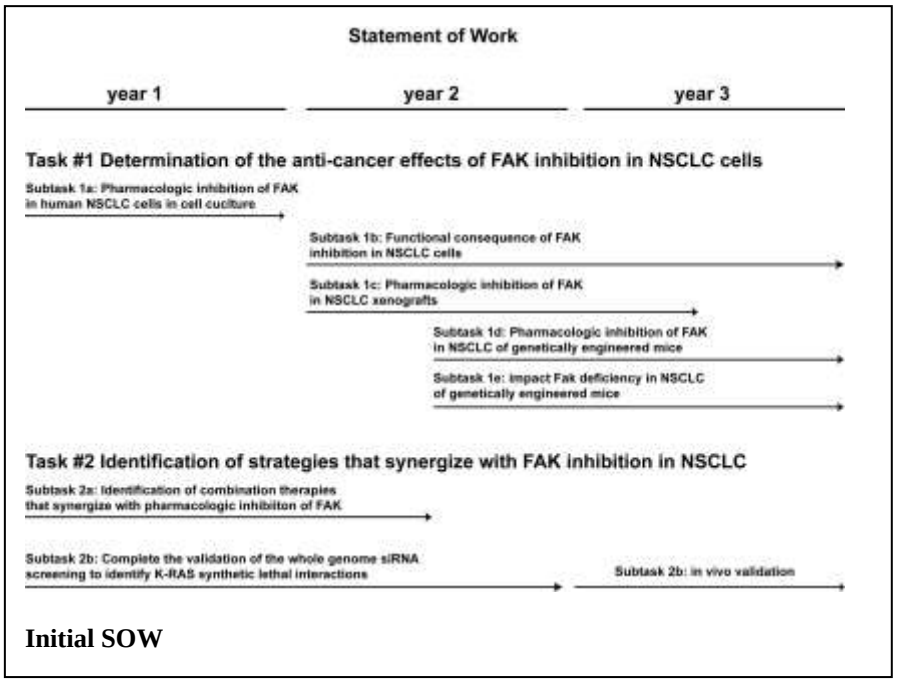
4.4. What was the impact on society beyond science and technology?

None to report

5. Changes/Problems.

There are no changes to the original SOW.

There are some delays in the original time-table, but the project is essentially on track.



Initial SOW

6. Products.

6.1. Publications, conference papers, and presentations.

Manuscripts:

Schuster K, Venkateswaran N, Rabellino A, Girard L, Pena-Llopis S, Scaglioni PP. Nullifying the CDKN2AB Locus Promotes Mutant K-ras Lung Tumorigenesis. Molecular cancer research : MCR. 2014;12(6):912-23. doi: 10.1158/1541-7786.MCR-13-0620-T. PubMed PMID: 24618618; PubMed Central PMCID: PMC4058359.

Manuscripts published during prior phases of the award:

Konstantinidou G, Ramadori G, Torti F, Kangasniemi K, Ramirez RE, Cai Y, et al. RHOA-FAK is a required signaling axis for the maintenance of KRAS-driven lung adenocarcinomas. Cancer Discov. 2013;3(4):444-57. doi: 10.1158/2159-8290.CD-12-0388. PubMed PMID: 23358651; PubMed Central PMCID: PMC3625467.

Abstracts at national of international meetings:

Inhibiting Focal Adhesion Kinase in Mutant KRAS NSCLC. Lung Cancer Summit. Ritz Carlton Dallas, Texas 12/15/2013.

Abstracts presented during prior phases of the award

1. G. Konstantinidou, G. Ramadori, F. Torti, C. Behrens, I. I. Wistuba, A. Heguy, D. Gerber, J. Teruya-Feldstein, P.P. Scaglioni. Identification of Focal Adhesion Kinase (FAK) as a Therapeutic Target in KRAS Mutant Lung Cancer. Mechanisms and Models of Cancer. Salk Institute, La Jolla, CA. August 7-10 2013. **Note that this abstract was selected for oral presentation.**

2. K. Schuster, N. Venkateswaran, S. Peña-Llopis and P.P. Scaglioni. Loss of the CDKN2AB Tumor Suppressor Locus Promotes Mutant KRAS Lung Tumorigenesis. Mechanisms and Models of Cancer. Salk Institute, La Jolla, CA. August 7-10 2013.

3. J.D. Constanzo, A. Rabellino, G. Konstantinidou, K. Schuster and P.P. Scaglioni. SUMO Ligase PIAS1: An Unexpected Role In Cancer Cell Initiation And Survival. Mechanisms and Models of Cancer. Salk Institute, La Jolla, CA. August 7-10 2013.

P.P. Scaglioni. Identification of Focal Adhesion Kinase (FAK) as a therapeutic target in KRAS mutant lung cancer. Lung Spore Workshop. National Cancer Institute., Rockville, MD. July 11-12, 2013. **Note that this abstract was selected for oral presentation.**

Presentations at Academic Institutions. Presenter, Pier Paolo Scaglioni, MD.

None this year on this specific topic

Presentations to academic institutions during prior phases of the award

Metabolic and Signaling Vulnerabilities of KRAS-driven lung adenocarcinoma. European Institute of Oncology, Milano, Italy. Grand Rounds. 1/22/2013.

Metabolic and Signaling Vulnerabilities of KRAS-driven lung adenocarcinoma. University of California, Division of Hematology/Oncology. Irvine, CA. 1/31/2013.

Metabolic and Signaling Vulnerabilities of KRAS-driven lung adenocarcinoma. Division of Hematology/Oncology. UT Health Science Center. San Antonio, TX. 2/21/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. Indiana University Medical Center. Indianapolis, IN. 4/18/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. Vanderbilt University Medical Center. Nashville. TN. 5/14/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. Case Comprehensive Cancer Center. Cleveland, OH. 5/22/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. City of Hope, Duarte, CA. 10/04/2013.

6.2 Website(s) or other Internet site(s)

NA

6.3 Technologies or techniques

We are generating genetically engineered lung cancer models: we will make them available to the scientific community as detailed in the appropriate section of the funded grant.

6.4 Inventions, patent applications, and/or licenses

NA

6.5 Other Product

NA

7. Participants and other collaborating organizations.

7.1. what individuals have worked on the project?

Name:	Mahesh Padanad
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Execution of Cell biology Experiments
Funding Support:	CPRIT Institutional Training Grant (since 3/2014)

Name:	Ke-Jing
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Execution of Cell biology Experiments

Funding Support:	Visiting Scholar supported by the Sun Yat-sen University, Guangzhou, China.

Name:	Ke-Jing
Project Role:	Research technician II
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Management of mouse colony and execution of experiments with mouse lung cancer models
Funding Support:	DOD

7.1. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The following are the research funds received by Dr. Scaglioni, the PI of this project.

Note that the first two grants are new

1. RP140672 (Scaglioni) 9/1/2014-8/31/2017 2.40 cal. month
Cancer Research Institute of Texas
Individual Investigator Research Award
Title: Mutant KRAS reprograms lipid metabolism exposing beta-oxidation as a novel therapeutic target in lung cancer lung cancer.
Major goal: with this grant we propose to determine the role of fatty acid beta-oxidation in metabolic reprogramming of mutant KRAS lung cancer cells.
Role: PI

2. 5P50 CA70907-15 (Minna) 9/1/2014-8/31/2019 0.60 cal. month
NIH/NCI
University of Texas SPORE (Special Program of Research Excellence) in Lung Cancer

The overall goal is the translation of findings to and from the laboratory and the clinic to result in improvement in the diagnosis, treatment, and prevention of lung cancer. It is a joint effort of UTSW and MDACC.
Role: Co-PI of Project #3 "Preclinical Development and Clinical Testing of MEK and PI3K Targeted Therapy for KRAS-mutant NSCLC as a Method of Radiosensitization and Metastasis Inhibition".

3. RO1 CA 137195A1 (Scaglioni) 7/1/2009-5/31/2015 (NCE) 2.40 cal. month
NIH/NCI
Title: Characterization and Drug Targeting of the PML Tumor Suppressor in Lung Cancer.
Major Goal: this grant proposes studies to identify the mechanisms that mediate PML tumor suppressive function with an approach that integrates a biochemical and molecular approach with studies in mouse models of lung cancer.
Role: PI

4. W81XWH-12-1-0210 (Scaglioni) 9/1/2012-8/31/2015 3.00 cal. month
CDMRP LCRP Investigator-Initiated Research Award
Title: Deconstruction of oncogenic K-RAS signaling reveals focal adhesion kinase as a novel therapeutic target in NSCLC
Major Goal: with this grant we will characterize the role of FAK signaling in mutant K-RAS induced NSCLC tumorigenesis

Role: PI

5. TBG 13-068-01 (Scaglioni) 1/1/2013-12/31/2016 1.20 cal. month
American Cancer Society Scholar Award
Title: Identification of critical components of the K-RAS network in lung cancer
Major Goal: with this grant we will identify vulnerabilities of mutant K-RAS lung cancer.
Role: PI

6. RP101251 9/1/2012-9/2017 0.60 cal. month
Cancer Prevention and Research Institute of Texas
Title: Development of Nuclear Receptor and Coregulator Profiles for Diagnostic and Therapeutic for diagnostic and therapeutic (theragnostic) targeting of breast and lung cancers.
Major goal: this grant proposes to determine whether nuclear receptors and nuclear co-receptors can be used as risk stratifiers or therapeutic targets in lung and breast cancers.
Role: Collaborator

7.2. What other organizations were involved as partners?

None to report

8. Appendices: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

1. Manuscript by Schuster K et al. Molecular Cancer Research. 2014;12(6):912-23. doi: 10.1158/1541-7786.MCR-13-0620-T. PubMed PMID: 24618618; PubMed Central PMCID: PMC4058359.

2. CV of Dr. Pier Paolo Scaglioni.

9. References

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Molecular Cancer Research

Nullifying the *CDKN2AB* Locus Promotes Mutant K-ras Lung Tumorigenesis

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Oncogenes and Tumor Suppressors

Molecular Cancer Research

Nullifying the *CDKN2AB* Locus Promotes Mutant K-ras Lung Tumorigenesis

Katja Schuster[1,2], Niranjan Venkateswaran[1,2], Andrea Rabellino[1,2], Luc Girard[3,4], Samuel Peña-Llopis[1,2,5], and Pier Paolo Scaglioni[1,2]

Abstract

Lung cancer commonly displays a number of recurrent genetic abnormalities, and about 30% of lung adenocarcinomas carry activating mutations in the *Kras* gene, often concomitantly with inactivation of tumor suppressor genes $p16^{INK4A}$ and $p14^{ARF}$ of the *CDKN2AB* locus. However, little is known regarding the function of *p15INK4B* translated from the same locus. To determine the frequency of *CDKN2AB* loss in human mutant *KRAS* lung cancer, The Cancer Genome Atlas (TCGA) database was interrogated. Two-hit inactivation of *CDKN2A* and *CDKN2B* occurs frequently in patients with mutant *KRAS* lung adenocarcinoma. Moreover, *p15INK4B* loss occurs in the presence of biallelic inactivation of $p16^{INK4A}$ and $p14^{ARF}$, suggesting that *p15INK4B* loss confers a selective advantage to mutant *KRAS* lung cancers that are $p16^{INK4A}$ and $p14^{ARF}$ deficient. To determine the significance of *CDKN2AB* loss *in vivo*, genetically engineered lung cancer mouse models that express mutant *Kras* in the respiratory epithelium were utilized. Importantly, complete loss of *CDKN2AB* strikingly accelerated mutant *Kras*–driven lung tumorigenesis, leading to loss of differentiation, increased metastatic disease, and decreased overall survival. Primary mutant *Kras* lung epithelial cells lacking *Cdkn2ab* had increased clonogenic potential. Furthermore, comparative analysis of mutant *Kras;Cdkn2a* null with *Kras;Cdkn2ab* null mice and experiments with mutant *KRAS;CDKN2AB*–deficient human lung cancer cells indicated that *p15INK4B* is a critical tumor suppressor. Thus, the loss of *CDKN2AB* is of biologic significance in mutant *KRAS* lung tumorigenesis by fostering cellular proliferation, cancer cell differentiation, and metastatic behavior.

Implications: These findings indicate that mutant *Kras;Cdkn2ab* null mice provide a platform for accurately modeling aggressive lung adenocarcinoma and testing therapeutic modalities. *Mol Cancer Res; 12(6); 912–23. ©2014 AACR.*

Introduction

Lung adenocarcinoma, a leading source of cancer-related mortality worldwide (1), harbors recurrent genomic abnormalities that provide a framework for the selection of targeted therapeutic agents (2–4). For these reasons, intense research efforts are under way to characterize the biologic and therapeutic significance of recurrent genomic abnormalities in non–small cell lung cancer (NSCLC). Activating mutations of *KRAS* occur in approximately 30% of lung adenocarcinomas. KRAS is a small GTPase that regulates several oncogenic networks (5). Mutant *KRAS* plays a causative role in lung tumorigenesis; however, it is not sufficient for the induction of high-grade lung adenocarcinomas in the absence of cooperating mutations that often involve the *CDKN2AB* locus (6–9).

The *CDKN2AB* locus encodes for several tumor suppressors. *CDKN2A* contains $p16^{INK4A}$ and $p14^{ARF}$ ($p19^{Arf}$ in mice), while *CDKN2B* contains $p15^{INK4B}$. Both $p16^{INK4A}$ and $p15^{INK4B}$ promote G_1 cell-cycle arrest by inhibiting the CDK4/CDK6 retinoblastoma family of tumor suppressors, whereas $p14^{ARF}$ upregulates TP53 by inactivating its negative regulator MDM2 (10, 11). Both $p16^{INK4A}$ and $p15^{INK4B}$ are highly similar and appear to have originated from gene duplication (12–14), whereas $p14^{ARF}$ gene expression is initiated from an exon intercalated between $p16^{INK4A}$ and $p15^{INK4B}$ and an alternative reading frame of exon 2 and 3 of $p16^{INK4A}$ (refs. 14, 15; Fig. 1A). Several studies established that $p16^{INK4A}$ and $p14^{ARF}$ are bona fide tumor suppressors *in vivo*, including in mutant *KRAS* lung adenocarcinomas (6, 9, 15–17).

Despite convincing biochemical evidence that $p15^{INK4B}$ is part of the TGF-β signaling pathway, much less is known regarding its tumor suppressor function *in vivo* (18). For

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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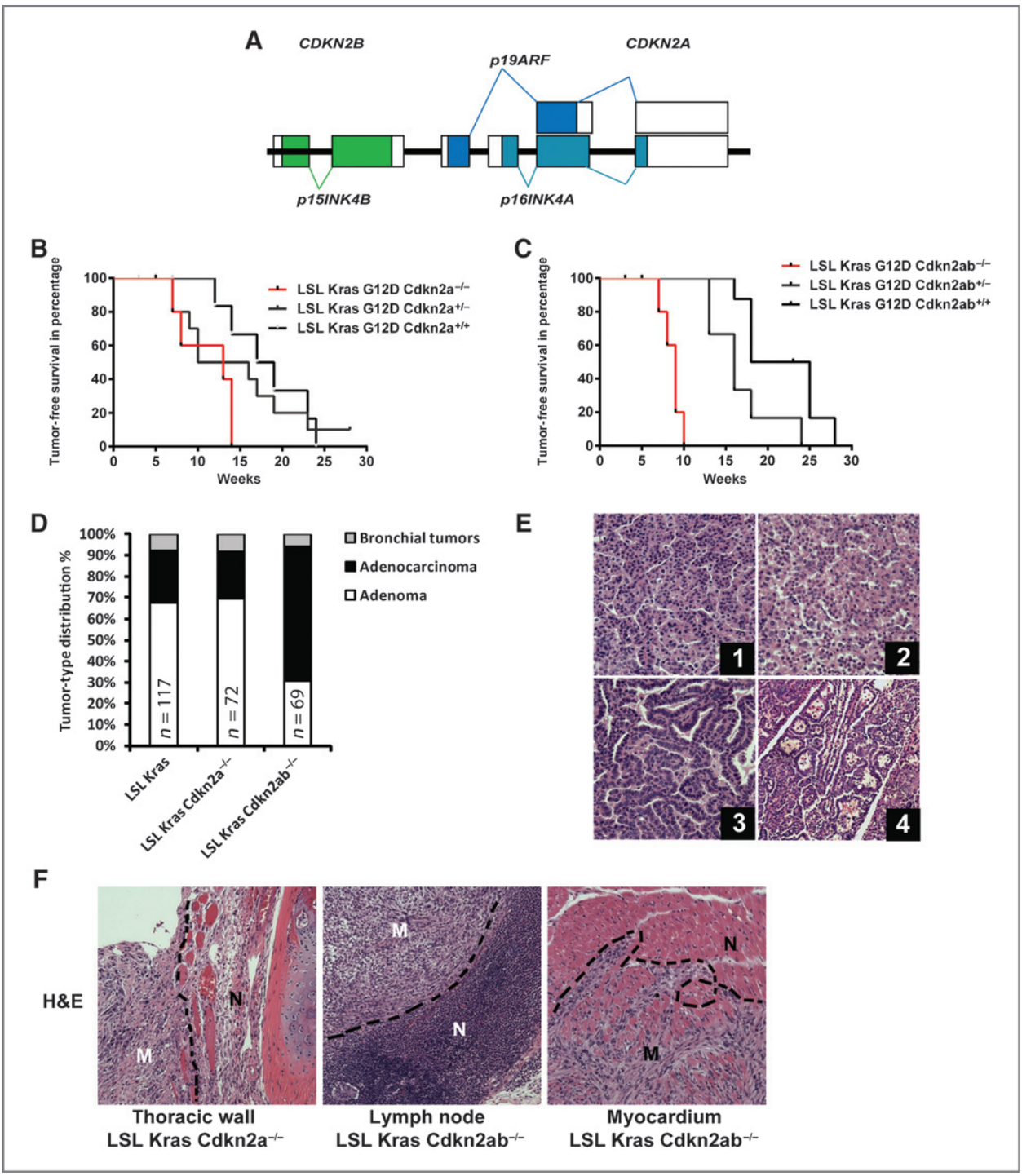

Figure 1. Loss of *Cdkn2ab* results in aggressive lung cancer in an oncogenic *Kras* conditional mouse. A, schematic of the *Cdkn2ab* locus in the mouse (not to scale). B, Kaplan–Meier survival curve of mice with the indicated genotypes ($n = 8$; $P = 0.024$, log-rank, Mantel–Cox, wild-type versus *Cdkn2a* null). C, Kaplan–Meier survival curve of mice with the indicated genotypes. Note that while *LSL-Kras;Cdkn2ab*$^{+/+}$ mice have a half-life of 21.5 weeks ($n = 8$), *LSL-Kras;Cdkn2ab*$^{-/-}$ mice ($n = 7$) have their half-life reduced to 9 weeks ($P < 0.0001$, log-rank, Mantel–Cox). D, Histogram of tumor type distribution of conditional mice with the indicated genotypes. Numbers of tumors counted are indicated. E, representative images of lung cancers stained with hematoxylin and eosin (H&E). (1) Lung adenoma of *LSL-Kras* and (2) of *LSL-Kras;Cdkn2a*$^{-/-}$ mice. (3) Adenocarcinoma of *LSL-Kras;Cdkn2ab*$^{-/-}$ mice and (4) bronchial tumor of *LSL-Kras;Cdkn2ab*$^{-/-}$, magnification 200 fold. F, metastases in the indicated genotypes and anatomic location. M, metastatic tissue; N, normal tissue. H&E staining, magnification $\times$100.

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example, *p15*^{INK4B} is rarely mutated independently of the other *CDKN2AB* genes (14). Furthermore, in the absence of other mutations, *p15*^{INK4B} null mice have only a mild tumor predisposition (19). However, Krimpenfort and colleagues demonstrated that *Cdkn2ab* null mice are tumor-prone and develop an expanded tumor spectrum as compared with *Cdkn2a* null mice (20). This study led to the conclusion that p15^{INK4B} provides a tumor-suppressive function that is critical in the absence of p16^{INK4A} and p19^{ARF} (20). It is unknown whether p15^{INK4B} has a tumor-suppressive role in other tumor models or whether its status influences tumorigenesis driven by activating mutations of proto-oncogenes commonly occurring in human cancer. For instance, even though *p15*^{INK4B} loss has been reported to occur in lung adenocarcinoma, its biologic significance has yet to be established in this context (2, 21, 22).

In this article, we study the significance of *Cdkn2ab* deficiency in the biology of mutant *Kras* lung adenocarcinoma with two genetically engineered mouse models that express mutant *KRAS* in the respiratory epithelium and human lung cancer cell lines. Furthermore, we determined the mutation frequency and pattern of the *CDKN2A* and *CDKN2B* loci by analyzing The Tumor Cancer Genome Atlas database. Our data indicate that *p15*^{INK4B} provides a tumor-suppressive function in mutant *KRAS* lung tumorigenesis.

Materials and Methods

Plasmids and lentiviral particle production

The cDNA of murine *Cdkn2b* (clone ID 3495097) was obtained from Open Biosystems (Thermo Scientific) and cloned into pLVX-tight-puro (Clontech Laboratories). Recombinant lentiviral particles were generated in 293T cells according to manufacturer's procedures.

Mouse models and tumor burden assessment

Tet-op-*Kras*;CCSP-*rtTA* were obtained from H.E. Varmus (6), *HIST1H2BJ-GFP* mice were from the Jackson Laboratory (23), *Ink4ab*$^{-/-}$ mice (hereafter named *Cdkn2ab*$^{-/-}$) were provided by Dr. Anton Berns (The Netherlands Cancer Institute, Amsterdam, the Netherlands), *LSL-Kras*^{G12D} mice were from the National Cancer Institute (NCI) mouse repository (24) and *Ink4a/Arf*$^{flox/flox}$ mice were from the Jackson Laboratory (25). Mice were maintained in a mixed background (FVB/N/CD-1). Experiments were performed with F3 generation progeny or later progenies, and comparisons were made with littermates. We obtained lung-specific *KRAS* expression at 4 weeks of age either by feeding mice with doxycycline-implemented food pellets (Harlan Laboratories) or by intratracheal administration of Adenovirus-Cre at 8 weeks of age (University of Iowa, Gene transfer Vector Core; refs. 6, 26). We administered doxycycline at 4 weeks of age to attempt to develop lung tumors before the development of tumors involving other organs, a common occurrence in *Cdkn2ab* null mice. All animal studies were completed according to the policies of the University of Texas Southwestern Institutional Animal Care and Use Committee. We used digital quantification of the area occupied by tumors to the area of total lung using the NIH ImageJ (v1.42q) software.

Primary cultures and colony formation assays

Single-cell suspensions of primary respiratory cells were sorted with a MoFlo cell sorter to select and plate GFP fluorescent cells on either irradiated or mitomycin C–treated mouse embryonic fibroblasts (MEF).

Cell lines

Human NSCLC cell lines H460 and A549 were from the Hamon Center cell line repository (UT Southwestern Medical Center, Dallas, TX).

Quantitative real-time PCR

We extracted RNA using the RNeasy Kit (Qiagen) and generated cDNA using iScript cDNA kit (Bio-Rad). For quantitative real-time PCR (qRT-PCR), we used iTaq SYBR green supermix with ROX (Bio-Rad).

Western blotting

We used the following antibodies: anti-PARP (Cell Signaling Technology), anti-HSP90 (BD Biosciences), anti-p15 (Sigma), and anti-phospho-H3 (Millipore).

Immunohistochemistry

We used the following antibodies: GFP (Chemicon), phospho-S6 ribosomal protein, HMG2A and phospho-Erk (Cell Signaling Technology), ALDH1A1 (Abcam), phospho-H3 (Millipore), NKX2-1 (Santa Cruz Biotechnology), SP-C (Chemicon), and CCSP (Santa Cruz Biotechnology). An HMG2A staining scoring system was used as follows: tumors were considered negative if no stain was visible, positive in few cells, positive in clusters of cells, and positive if >50% of tumor cells scored HMG2a positive.

Analyses of the TCGA database

Information regarding mutation, segmented copy number, methylation, and clinical data of primary lung adenocarcinomas were obtained from TCGA data portal (https://tcga-data.nci.nih.gov/tcga) on June 8, 2013. Only nonsilent mutations were considered as described (27). Kaplan–Meier was used to estimate the survival curves and comparisons were performed using the log-rank test using SPSS Statistics 17.0. One-way ANOVA followed by Tukey *post hoc* test was used to compare the pathologic stage among groups.

Copy number and microarray analysis of lung cancer cell lines

Most of the *KRAS* and *CDKN2A* mutations data were obtained from the COSMIC database (Sanger Institute, United Kingdom), the literature, or from our unpublished whole-exome sequencing data (28). mRNA expression microarrays were performed by us with Illumina HumanWG-6 V3 as previously described (29). They were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession GSE32036. Whole-genome Single-nucleotide polymorphism (SNP) profiling was done as previously described (30).

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Results

Cdkn2ab deficiency accelerates mutant *KRAS* lung tumorigenesis in mice

To characterize the effect of deficiency of genes encoded by *Cdkn2ab* on lung tumorigenesis (Fig. 1A), we generated compound mutant mice expressing mutant *KRAS* in the respiratory epithelium. We crossed *LSL-Kras*^{G12D} mice (*LSL-Kras* mice), which encode a latent mutant *Kras* gene, with mice harboring conditional alleles of *Cdkn2a* or *Cdk2ab* (20, 24, 25). We achieved lung-specific tumor development by intratracheal delivery of adenovirus encoding for the Cre recombinase.

The overall median survival of *LSL-Kras*;*Cdkn2a*$^{-/-}$ was significantly decreased as compared with their littermates with a wild-type *Cdkn2a* locus, whereas *LSL-Kras*;*Cdkn2a*$^{+/-}$ had a trend toward an intermediate survival between *Cdkn2a* wild-type and null genotypes (Fig. 1B). The survival of *LSL-Kras*;*Cdkn2ab*$^{-/-}$ mice was significantly decreased as compared with *LSL-Kras*, *LSL-Kras*;*Cdkn2ab*$^{+/-}$ or *LSL-Kras*;*Cdkn2a*$^{-/-}$ mice (Fig. 1C). These findings demonstrate that complete loss of *Cdkn2ab* significantly promotes mutant KRAS lung tumorigenesis.

Mutant *KRAS* induces high-grade and metastatic lung adenocarcinomas in *Cdkn2ab* null mice

At the time of death, the majority of lung parenchyma was occupied by lung tumors in every genotype (Supplementary Fig. S1A–S1C). This finding suggests that death is caused by respiratory compromise.

We determined genotype-specific histologic features: lungs of *LSL-Kras* and *LSL-Kras*;*Cdkn2a*$^{-/-}$ mice contained predominantly lung adenomas (Fig. 1D and E, panel 1 and 2) and occasional carcinomas with nuclear atypia (not shown). In contrast, *Cdkn2ab* null mice carried primarily high-grade lung adenocarcinomas consisting of atypical nuclei arranged in papillary structures (Fig. 1E, panel 3). Moreover, we detected tumors arising from the bronchial epithelia in all three genotypes (Fig. 1D and E, panel 4).

We observed that 44% of *LSL-Kras*;*Cdkn2a*$^{-/-}$ mice carried metastatic lesions in mediastinal lymph nodes, or the thoracic wall (Fig. 1F; Supplementary Table S1). This metastatic behavior was even more pronounced in *LSL-Kras*;*Cdkn2ab*$^{-/-}$ mice, where 89% of the mice carried lung tumors that metastasized to mediastinal lymph nodes and the myocardium (Fig. 1F; Supplemental Table S1). These finding indicate that *p15*^{Ink4b} deficiency leads to high-grade lung adenocarcinomas with aggressive behavior, including the property to generate locoregional metastasis.

Kras;Cdkn2ab null lung tumors display upregulation of proliferative markers

We determined the level of activity of targets of KRAS, which have been implicated in tumorigenesis, and of the mitotic marker phospho-Histone 3 (p-H3). *LSL-Kras*;*Cdkn2a* null and *LSL-Kras*;*Cdkn2ab* null tumors were diffusely positive for p-S6 and p-Erk1/2, with more intense staining in tumor segments with atypical nuclei and papillary morphology, whereas *LSL-Kras*;*Cdkn2ab*$^{+/+}$ lung tumors were faintly positive for p-S6 and p-Erk1/2 (Fig. 2A).

The staining pattern of p-H3 was genotype dependent: *LSL-Kras*;*Cdkn2a*$^{-/-}$ tumors stained positive for p-H3 mostly at the tumor edge. In contrast, *LSL-Kras*;*Cdkn2ab*$^{-/-}$ tumors stained positive throughout the tumor mass. Furthermore, the number of mitotic cancer cells increases significantly in *LSL-Kras*;*Cdkn2ab*$^{-/-}$ tumors as compared with *Cdkn2a*$^{-/-}$ and *Cdkn2ab*$^{+/+}$ lung tumors (Fig. 2A and B). We conclude that the loss of *Cdkn2ab* promotes cellular proliferation and progression of KRAS-driven lung tumors.

Cdkn2ab deficiency leads to reduction of differentiation of KRAS^{G12D}-driven lung tumors

To further characterize the lung tumors that develop in *LSL-Kras;Cdkn2ab* null mice, we evaluated the lung epithelial marker NK2.1 homeobox 1 (NKX2-1) and chromosomal high motility group protein HMGA2, which define the status of differentiation of lung adenocarcinoma driven by mutant KRAS in the mouse (31, 32). NKX2-1 is a master regulator of lung differentiation, which is expressed in the majority of human lung adenocarcinomas (33). Human lung tumors that are negative for NKX2-1 are poorly differentiated, display high-grade histologic features, and an aggressive behavior (33–35). NKX2-1 restrains lung cancer progression by enforcing a lung epithelial differentiation program by repressing the chromatin regulator HMGA2 (31, 32).

We found that lung tumors of *LSL-Kras*;*Cdkn2ab*$^{+/+}$ and *LSL-Kras;Cdkn2a*$^{-/-}$ mice are predominately NKX2-1–positive and HMGA2-negative (Fig. 2C and D and Supplementary Fig. S2A). In contrast, about 21% of *LSL-Kras*;*Cdkn2ab*$^{-/-}$ tumors lose NKX2-1 completely (Supplementary Fig. S2A). In addition, about 73% of *LSL-Kras*;*Cdkn2ab*$^{-/-}$ tumors increase their number of HMGA2-positive cells (Fig. 2C–E and Supplementary Fig. S2B). Finally, we also determined that the metastases that developed in *Cdkn2a*$^{-/-}$ and in *Cdkn2ab*$^{-/-}$ lung tumors stain positive for HMGA2 (Supplementary Fig. S2C). This observation strongly suggests that HMGA2-positive lung tumors have metastatic potential.

These data indicate that complete loss of the *Cdkn2ab* locus significantly promotes lung cancer tumor progression in the mouse. These observations imply that p15^{INK4B} provides an important tumor suppressor function in opposing mutant KRAS tumorigenesis.

Transgenic mouse model of genetically labeled cancer-initiating cells

To characterize in better detail the biologic consequence of *Cdkn2ab* deficiency in oncogenic *Kras* lung adenocarcinoma, we generated mice that allow the tagging and isolation of lung cancer cells. We took advantage of *CCSP-rtTA*;*Tet-op-Kras* mice, which carry a transgene encoding *Kras*^{G12D} under the control of the tetracycline operator (*Tet-op-Kras*), and a transgene expressing the reverse tetracycline transactivator in the respiratory epithelium under the control of the Clara cell secretory protein promoter (*CCSP-rtTA*). *Tet-op-Kras*;*CCSP-rtTA* mice develop lung cancer with

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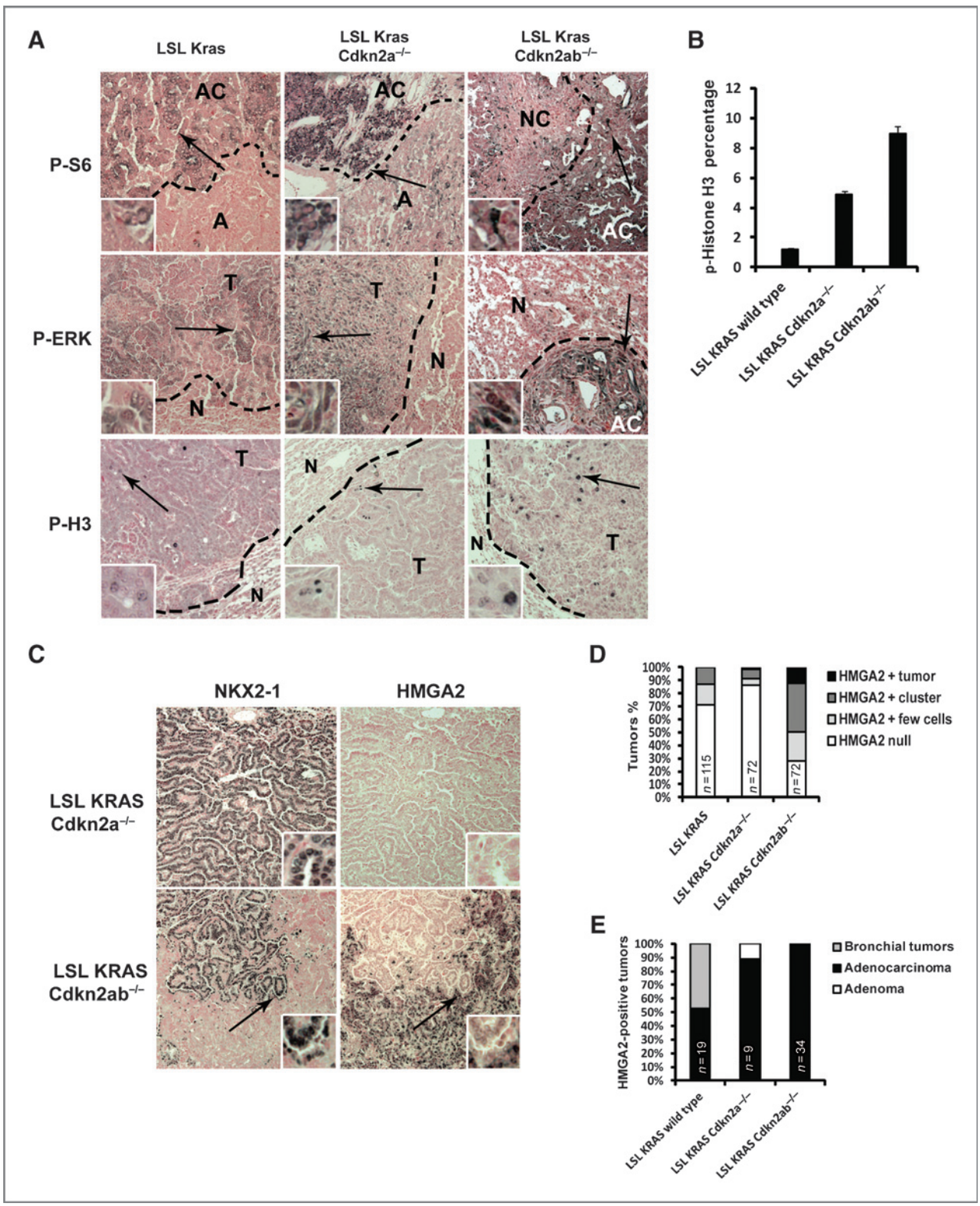

Figure 2. Loss of *Cdkn2ab* increases proliferation, and tumor progression in mutant *Kras* lung tumors. A, images of lung tumors stained with the indicated antibodies (×100). Antibody staining is shown in brown, nuclear fast red counter-stain is pink-red. Arrows show location of inlets. A, adenoma; AC, adenocarcinoma; T, tumor; N, normal tissue. B, histogram of the percentage of p-H3 positive cells lung tumors. C, Sections of lung tumors stained as indicated (×100). Arrows indicate the location of inlets. D, histogram representing percentage of lung tumors with the indicated HMGA2 staining pattern (refer to methods for scoring scale). E, anatomic location and classification of HMGA2-positive tumors. Number of tumors is indicated.

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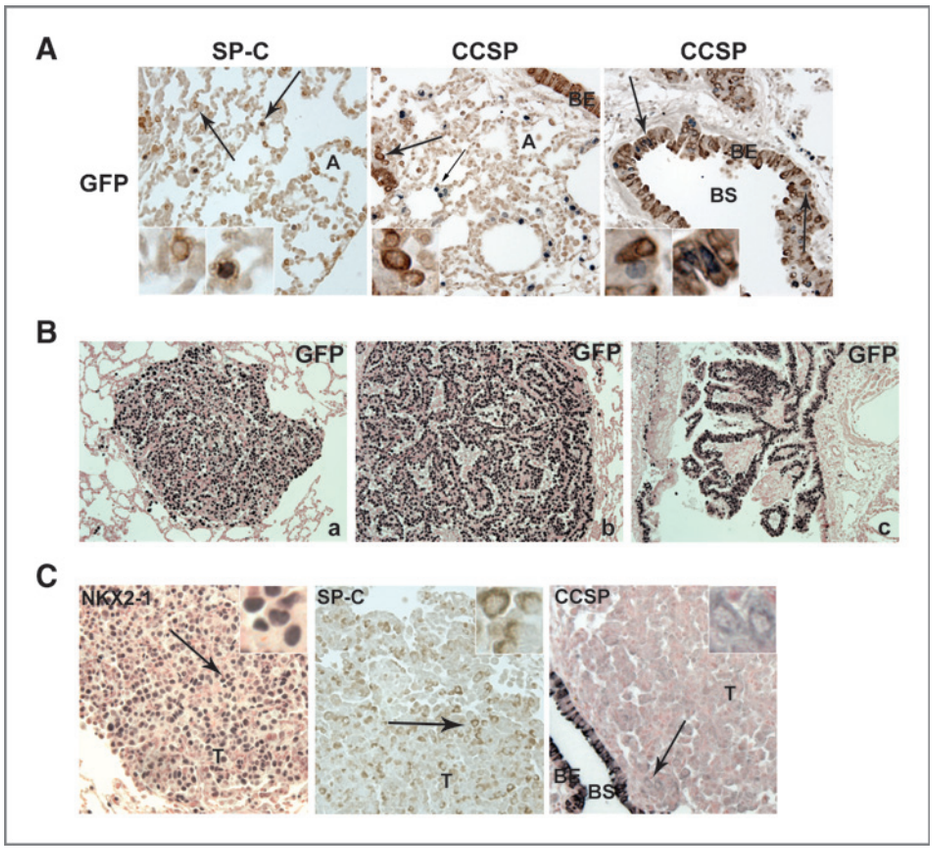

Figure 3. Lung epithelial cells and tumors genetically labeled by H2B-GFP. A, lung sections stained with the indicated antibodies. H2B-GFP–positive cells in the alveoli stain positive for SP-C, suggesting that they are alveolar type II pneumocytes. H2B-GFP staining: black gray, SP-C and CCSP staining: brown. Arrows indicated the location of the inlets. Magnification, ×200. A. B, immunohistochemistry of lung tumors from *Kras-GFP* mice stained as indicated: (a) adenoma, (b) adenocarcinoma, (c) bronchial tumor (×100). C, *Kras-GFP* tumors stained as indicated. Arrows indicate the location of the inlets. Images are enlarged 200-fold. CCSP-rtTA transgene is expressed, albeit at low level, in lung cancer cells. These data independently confirm the observations of Fisher et al. regarding the pattern of expression of CCSP-rtTA (6).

100% penetrance when continuously exposed to doxycycline (6).

To visualize mutant *KRAS*–expressing lung epithelial cells, we crossed the *Tet-op-Kras;CCSP-rtTA* strain with mice that carry a transgene encoding histone *H2B* fused to *GFP* (*H2B-GFP*) under the control of the tetracycline operator (tetO-*HIST1H2BJ-GFP*; ref. 23). We determined that upon exposure to food pellets impregnated with doxycycline, Tet-op-*Kras*,*CCSP-rtTA*;tetO-*HIST1H2BJ-GFP* (*Kras-GFP* mice) transgenic mice express *H2B-GFP* in lung epithelial cells (Supplementary Fig. S3A–S3C) with the characteristics of type II pneumocytes (Fig. 3A).

When exposed continuously to doxycycline, *Kras-GFP* mice develop mutant *KRAS* lung tumors that are also positive for H2B-GFP (Fig. 3B). Lung tumor cells showed predominantly nuclear NKX2-1 staining and cytoplasmic SP-C staining (Fig. 3C). Tumor cells were predominantly negative for CCSP but we detected faint CCSP staining in a portion of tumor cells as compared with the strong staining in the bronchiolar epithelial lining (Fig. 3C). We conclude that this mouse model allows successful H2B-GFP tagging of lung cancer cells.

Cdkn2ab deficiency promotes the development of high-grade non–small cell lung carcinomas

We determined the effect of *Cdkn2a* or *Cdkn2ab* deficiency on lung tumorigenesis in *Kras-GFP* mice. Significantly, *Kras-GFP*;*Cdkn2ab*$^{-/-}$ mice developed an increased number of tumors as well as an increased tumor burden 4.5 months after induction of mutant *KRAS* (Fig. 4A–C). In this mouse model, loss of *Cdkn2a* resulted in an intermediate number of tumors and tumor burden at 4.5 months (Supplementary Fig. S4A and S4B). We also observed an increase in tumor burden in *Kras-GFP;Cdkn2ab*$^{-/-}$ mice at time of death (Supplementary Fig. S4C). These results indicate that *Cdkn2ab* loss promotes both mutant KRAS tumor initiation and progression.

Tumors arose within the lung parenchyma and alveoli in all three genotypes (Fig. 4D); however, in this model only *Cdkn2ab* null mice generated tumors that arose from the bronchial epithelia (Fig. 4D, panels 4 and 9 and Supplementary Fig. S4D). This observation suggests that *Cdkn2ab* loss reduces the threshold to develop oncogenic *KRAS*-induced tumors in distal bronchioles. In addition, *Cdkn2a* or *Cdkn2ab* null mice developed lung tumors characterized by nuclear atypia and papillary structures (Fig. 4D, panels 2, 3, 7 and, 8; Supplementary Fig. S4D) that closely resembles aggressive human NSCLC (36, 37). Others and we described similar morphologic changes in lung adenocarcinomas of *Cdkn2a* null mice induced by the doxycycline-regulated mutant *Kras* transgene (6, 9, 38).

Lung tumors of *Cdkn2ab*-deficient mice show an increase in the number of cells stained positive for targets of activated KRAS, such as p-S6 ribosomal protein and p-ERK1/2 (Supplementary Fig. S4E). These findings suggest that

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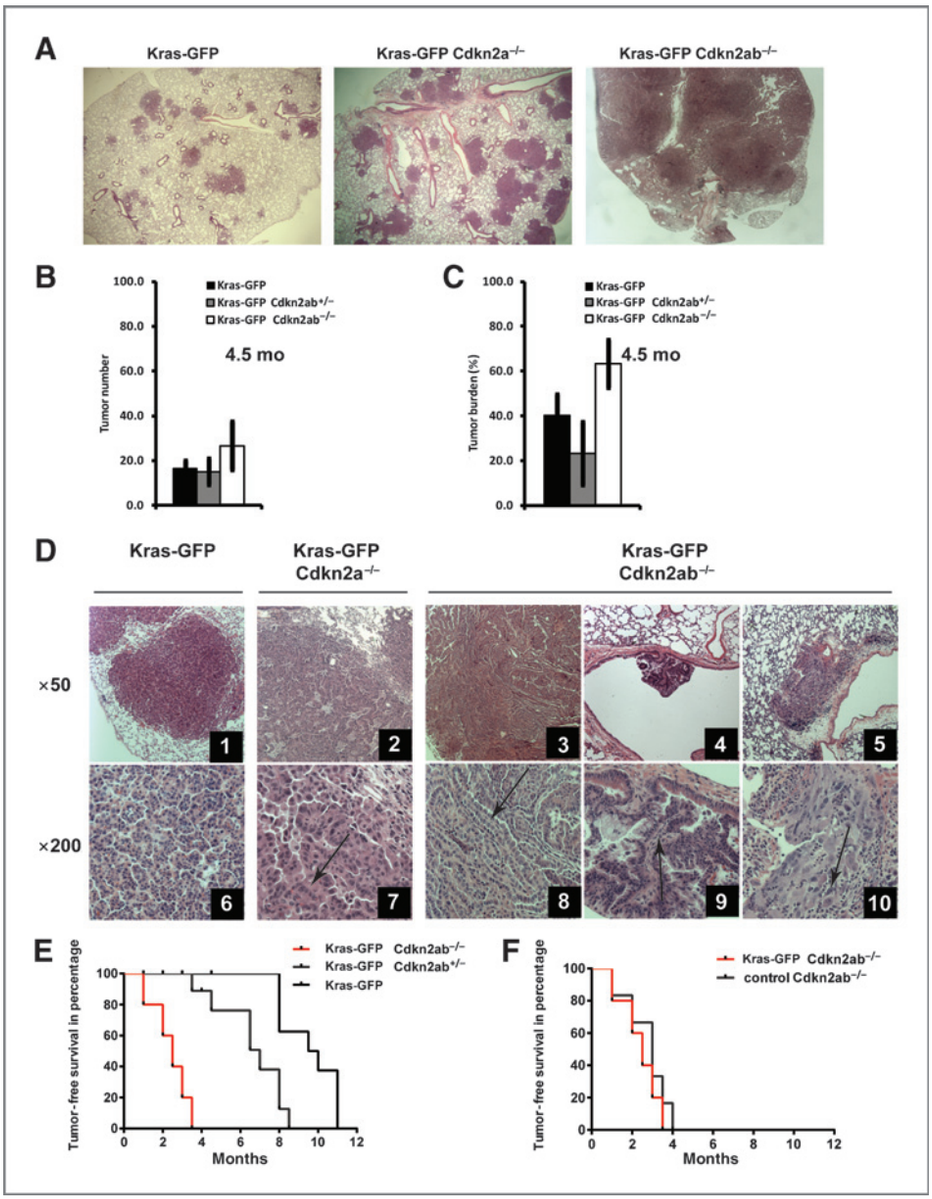

Figure 4. Loss of *Cdkn2ab* results in high-grade lung cancer and increased tumor burden. A, representative lung section of mice of the indicated genotypes 4.5 months after doxycycline exposure (×15 magnification). B and C, histograms show tumor number or burden in mice of the indicated genotype after 4.5 months of doxycycline. Note increased tumor number/burden in mutant *Kras-GFP;Cdkn2ab*$^{-/-}$ mice. D, lung tumors of the indicated genotypes. Arrows indicate areas with papillar features and atypical nuclei (panels 6, 7, 8, and 9) or with poor differentiation (panel 10).

Cdkn2ab deficiency leads to increased proliferative activity. These features reproduced the characteristics of lung tumors developed in *LSL-Kras;Cdkn2ab*$^{-/-}$ mice.

Kras-GFP;*Cdkn2ab*$^{+/+}$ mice had the longest survival, *Kras-GFP*;*Cdkn2a*$^{-/-}$ mice had an intermediate survival and *Kras-GFP*;*Cdkn2ab*$^{-/-}$ mice had the shortest survival among the three groups (Supplementary Fig. S4G). These differences, however, were due mainly to sarcomas and lymphomas, which preclude the assessment of lung cancer–specific mortality (Supplementary Fig. S4H and Supplementary Table S2).

H2B-GFP–positive epithelial cells form colonies *in vitro*

To evaluate the properties of lung epithelial cells marked by H2B-GFP, we obtained single-cell suspensions from the lungs of *Kras-GFP* and CCSP-*rtTA;*tetO-*HIST1H2BJ-GFP* control transgenic mice (control-GFP mice). We found that one of 85 H2B-GFP–positive oncogenic *KRAS*-expressing lung epithelial cells formed colonies, whereas only 1 of 150 GFP-positive, but wild-type *Kras* lung epithelial cells were able to do so (Supplementary Fig. S5A and S5B). We did not detect any difference in the number of lung epithelial cells present in colonies of either control or mutant *KRAS*–expressing GFP-positive cells (Supplementary Fig. S5C). This finding suggests that mutant *KRAS* promotes clonogenicity, but not cell proliferation under these conditions.

We were able to passage these cells up to 5 times onto feeder cultures, suggesting that they can be propagated *in vitro* and have short-term self-renewal abilities. Of note, similar colony formation ratios were reported for bronchoalveolar stem cells,

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putative epithelial stem cells present at the bronchoalveolar junction (39).

We were unable to propagate H2B-GFP–positive cells obtained from the respiratory epithelium beyond 5 passages due to loss of viability. In contrast, H2B-GFP–positive primary lung epithelial cells obtained from *Kras-GFP; Cdkn2a*$^{-/-}$ or *Kras-GFP;Cdkn2ab*$^{-/-}$ mice readily give rise to cell lines when grown as monolayer cultures in conventional tissue cultures plates and retain a subpopulation of H2B-GFP–positive cells for up to 20 passages (Supplementary Fig. S5D and S5E). Moreover, H2B-GFP–positive cells are positive for lung epithelial markers and the cancer stem cell marker ALDH1A1 (Supplementary Fig. S5F). As expected, only H2B-GFP–positive cells express mutant *Kras* (Supplementary Fig. S5G). Moreover, mutant *Kras* expression is doxycycline dependent and cannot be reinduced (Supplementary Fig. S5H). Taken together, these data suggest that deficiency of the genes of the *Cdkn2ab* locus promote immortalization of the respiratory epithelial cells.

Loss of *p15*^{INK4B} promotes mutant KRAS tumorigenesis in human lung cancer

To determine the frequency of the loss of genes of the *CDKN2AB* locus, we determined the mutation status of *p15*^{INK4B}, *p16*^{INK4A} and *p14*^{ARF} in a panel of 23 human NSCLC lines which express mutant KRAS. For this purpose, we used direct sequencing to detect point mutations and SNP analysis the detect gene copy number alteration (Table 1). We established that 6 of the cell lines carried homozygous deletions of *CDKN2AB* and 15 cell lines carried at least heterozygous deletion of *CDKN2AB*. Only two cell lines (HCC44 and H1155) did not lose a single copy of the *CDKN2AB* allele. We validated nullyzygosity of the *CDKN2AB* locus in A549 and H460 by qRT-PCR (Fig. 5A). These findings indicate that loss-of-function of all three tumor suppressors of *CDKN2AB* is a common event in human lung adenocarcinoma.

We next analyzed patient data from The Cancer Genome Atlas (TCGA) database. Data with mutation, copy number, and methylation was available for 323 lung adenocarcinoma patients and showed that 30% ($n = 96$) had oncogenic *KRAS* mutations. In addition, 51 of the 96 patients (53%) with *KRAS* mutations also had two-hit *CDKN2A* inactivation by mutation, chromosomal deletion, and/or promoter methylation. Of these patients, 17 (18%) also showed inactivation in the *CDKN2B* locus by homozygous deletions. None of the patients in this database showed inactivation of *CDKN2B* but not of *CDKN2A*. These data indicate that the loss of the *CDKN2AB* locus is a common occurrence in human NSCLC (Fig. 5B). Notably, deficiency of *CDKN2A* is associated with higher pathologic stage (Supplementary Table S3), poorer overall survival (Fig. 5C), and progression-free survival (Fig. 5D) in patients with mutant *KRAS* lung adenocarcinoma. While there is a tendency to poorer progression-free survival with inactivation of *CDKN2B* in addition to *CDKN2A* ($P = 0.19$), this difference was not significant. Anyway, inactivation of either *CDKN2A* or *CDKN2AB* is associated with poorer progression-free survival and overall survival (Supplementary Fig. S6A and S6B) independently of the *KRAS* mutation status.

Table 1. *CDKN2AB* status of *KRAS* mutant NSCLC lines

Cell line	Tumor subtype	CDKN2AB status	KRAS
A549	Adenocarcinoma	HOMO	MUT
H460	Large cell	HOMO	MUT
H1944	Adenocarcinoma	HOMO	MUT
HCC1171	NSCLC	HOMO	MUT
HCC4017	Large cell	HOMO	MUT
HOP-62	Adenocarcinoma	HOMO	MUT
H23	Adenocarcinoma	HET	MUT
H157	Squamous	HET	MUT
H358	Adenocarcinoma	HET	MUT
H441	Adenocarcinoma	HET	MUT
H1355	Adenocarcinoma	HET	MUT
H1373	Adenocarcinoma	HET	MUT
H1734	Adenocarcinoma	HET	MUT
H2009	Adenocarcinoma	HET	MUT
H2122	Adenocarcinoma	HET	MUT
H2347	Adenocarcinoma	HET	MUT
H2887	NSCLC	HET	MUT
HCC366	Adenosquamous	HET	MUT
HCC461	Adenocarcinoma	HET	MUT
HCC515	Adenocarcinoma	HET	MUT
Calu-6	Adenocarcinoma	HET	MUT
H1155	Large cell	WT	MUT
HCC44	Adenocarcinoma	WT	MUT

NOTE: CDKN2AB mutation status is indicated as WT, wild type; HOMO, homozygous loss; HET, heterozygous loss; MUT–KRAS mutation. Pathologic classification of cell lines is indicated as it was given at the time the cell lines were raised.

p15^{INK4B} expression promotes growth arrest in human lung cancer cells

To determine the functional effect of p15^{INK4B} expression *in vitro*, we ectopically expressed 15^{INK4B} in mutant *KRAS* human lung cancer cell lines that also carry homozygous deletions of the *CDKN2AB* locus. We determined that reintroduction of 15^{INK4B} leads to an increase in the percentage of A549 and H460 cells in the G_1 phase of the cell cycle (Fig. 6A). Furthermore, we noticed a significant increase of the percentage of subdiploid cells in A549 cells, which indicate the presence of apoptosis. Western blot analysis confirmed that expression of *p15*^{INK4B} downregulates the phosphorylation of mitotic marker p-H3 (Ser 10; Fig. 6B). These results suggest that the expression of p15^{INK4B} promotes a tumor suppressor program that counteracts the proliferative effects of mutant *KRAS* in NSCLC cells.

Discussion

Recent technical advancements have allowed a systematic evaluation of statistically recurrent somatic mutations in

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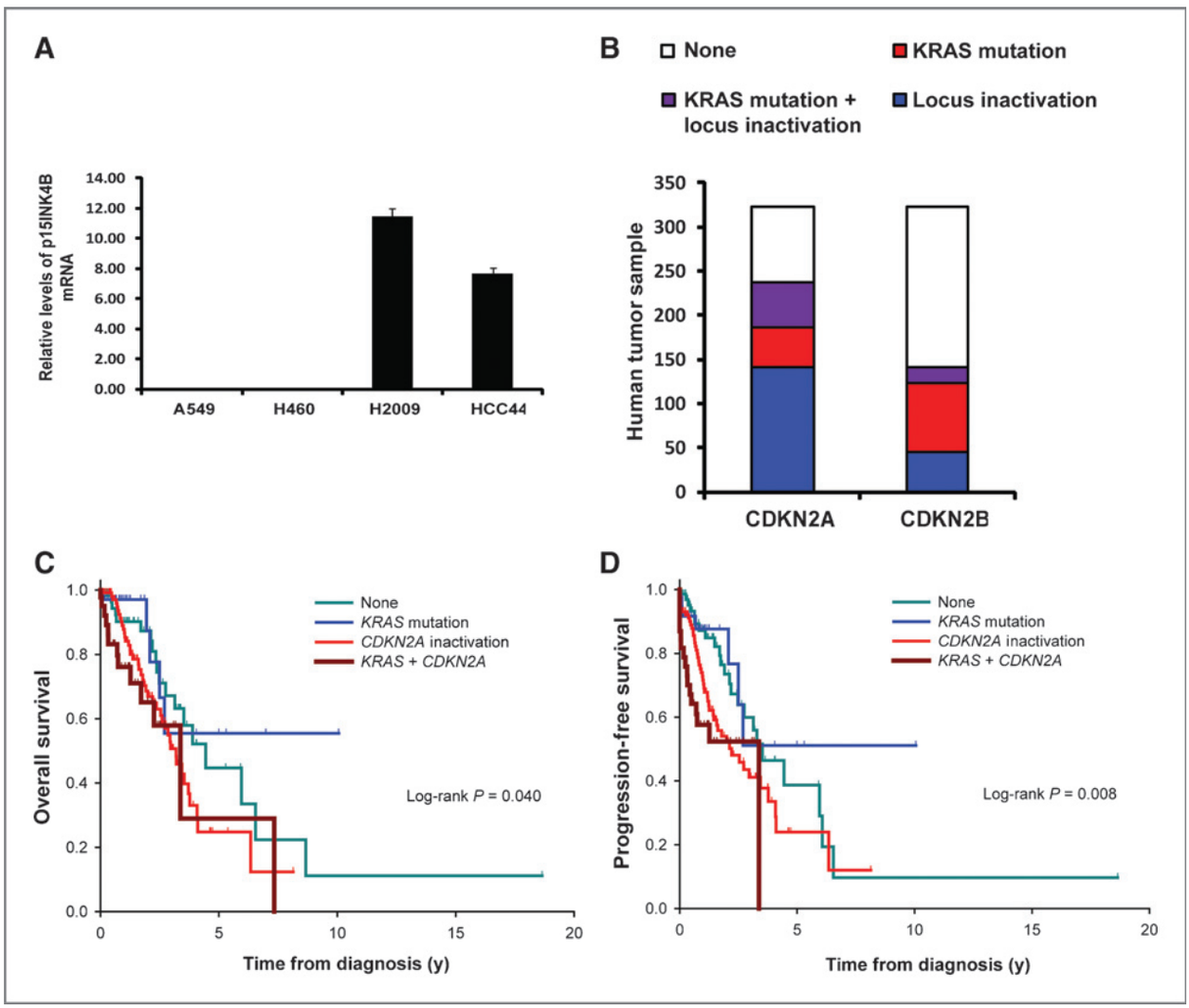

Figure 5. *CDKN2AB* status in human mutant *KRAS* lung adenocarcinoma. A, histogram of $p15^{INK4B}$ mRNA in NSCLC cell lines normalized to *GAPDH*. B, histogram shows the frequency of *KRAS* mutations and *CDKN2A* or *CDKN2AB* inactivation in lung adenocarcinoma in the TCGA data set. C, overall survival and D, progression-free survival curves of patients with lung adenocarcinoma carrying mutant *KRAS* and/or inactivation of *CDKN2A*.

NSCLC (2, 3). This data set holds the promise to better understand lung tumorigenesis and to identify novel treatments for patients with lung cancer.

In this study, we show that genetic inactivation of the *CDKN2A* and *CDKN2B* loci is a common event in mutant *KRAS* lung adenocarcinoma. By analyzing the TCGA database, we determined that inactivation of *CDKN2A* by either somatic mutation, chromosomal deletion, and/or promoter hypermethylation, and inactivation of *CDKN2B* by homozygous deletion occur in about 50% and 20% of human lung adenocarcinomas, respectively. Previous reports did not have sufficient power to determine whether inactivation of the genes of *CDKN2AB* occurs with significant frequency in mutant *KRAS* lung cancer (2, 40). By analyzing the large and comprehensive TCGA sample cohort, it is apparent that inactivation of *CDKN2AB* occurs at a significantly higher frequency than previously noted. Furthermore, we noticed that loss of *CDKN2B* rarely, if ever, occurs in the absence of *CDKN2A* somatic inactivation. Our analysis of the TCGA data set also indicates that loss of *CDKN2A* is associated with higher stage, higher incidence of metastasis, and consequently, decreased overall survival in patients with mutant *KRAS* lung cancer. These findings have significant clinical implications since they suggest that mutant *KRAS* lung cancers with two-hit inactivation of *CDKN2A* identify a subset of patients with high-risk disease. In this regard, it is of interest that these patients could benefit from treatment with focal adhesion kinase inhibitors (9).

We recently reported that expression of oncogenic *KRAS* in the respiratory epithelium significantly reduced the longevity of $p16^{Ink4a}/p19^{Arf}$ null mice (9). We observed a further increase in lung cancer–mediated mortality in mutant *Kras* mice conditionally deficient in the entire *Cdkn2ab* locus. This observation suggests a model whereby $p15^{INK4B}$ loss

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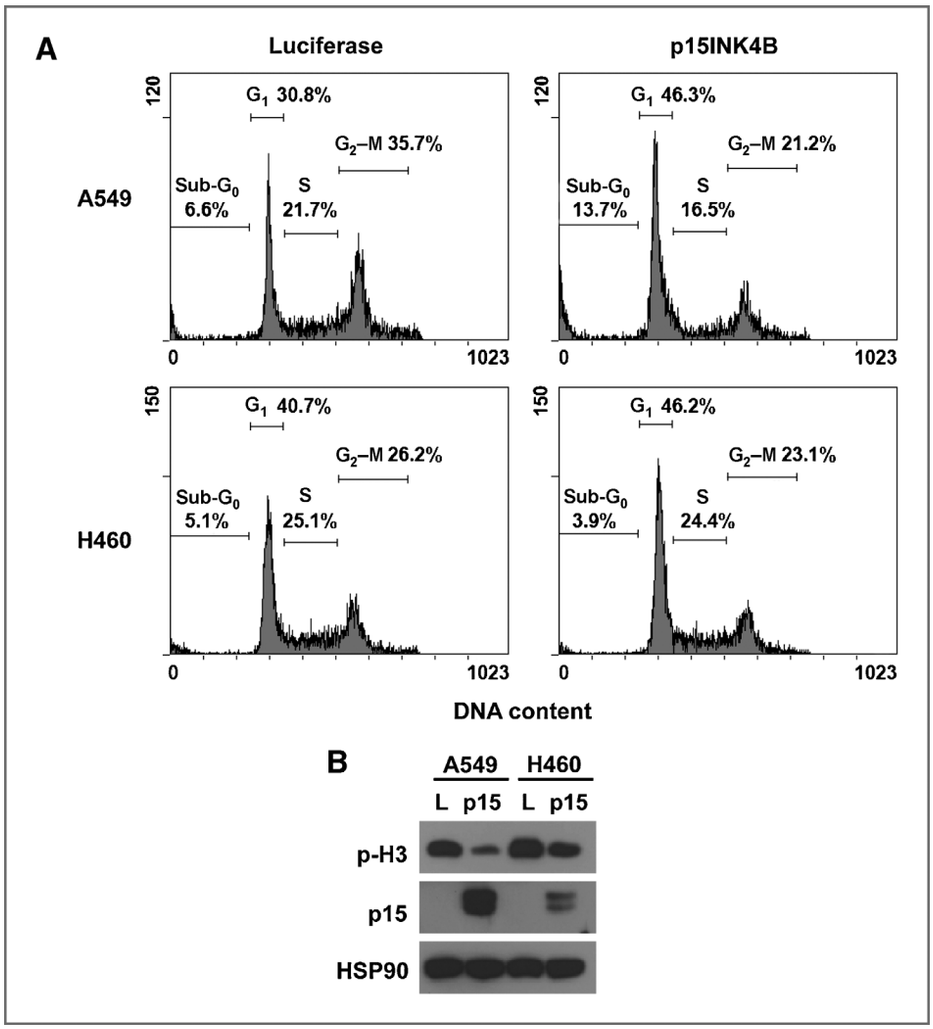

Figure 6. Reintroduction of $p15^{INK4B}$ in *CDKN2AB*-deficient lung cancer cell lines causes in growth arrest and apoptosis. A, cell-cycle analysis of A549 and H460 cells after 72 hours of luciferase or $p15^{INK4B}$ expression. Both cell lines show an increase in the G_1 phase of the cell cycle. A549 cells also showed an increase in sub-G_1 fraction, suggesting that a portion of cells undergoes apoptosis. B, Western blot analysis of A549 and H460 cells transduced as indicated. L, luciferase.

confers a selective advantage to tumor cells that have lost $p16^{INK4A}$ and $p14^{ARF}$. However, we did not detect a worse clinical outcome in patients with mutant *KRAS* lung cancers with loss of *CDKN2AB* as compared with patients with lung cancers mutant for *KRAS* and *CDKN2A*. We reason that this observation could be due to several variables, including insufficient sample size of the TCGA data set, or the presence of concurrent mutations that affect the phenotype of human cancer that are not present in genetically engineered mice.

We determined histologically that *Cdkn2a* deficiency leads to a higher percentage of high-grade lung tumors in Tet-o-*Kras* mice than in *LSL-Kras* mice, while the lung tumor phenotype of mutant *Kras;Cdkn2ab* null mice is consistent between the two mouse models. It is likely that these differences are either due to the strength of the promoters used to express mutant *Kras* or to the fact that we exposed mice to doxycycline at 4 weeks of age to attempt to obtain lung tumors before the emergence of tumors in other sites. We reason that *LSL-Kras* mice, owing to the fact that mutant *Kras* is expressed from the endogenous promoter, model more closely mutant *KRAS* tumorigenesis.

Our *in vivo* cell-tracking experiments are consistent with a recent report indicating that a subset of alveolar type II cell coexpressing CCSP and SP-C give rise to lung adenocarcinomas upon mutant KRAS expression (41). The observation that loss of *Cdkn2ab* promotes the clonogenic ability of lung epithelial cells and their ability to readily give rise to cell lines supports the notion that *Cdkn2ab* regulates cell proliferation. This assertion is further supported by our observation that reintroduction of $p15^{INK4B}$ induces antiproliferative effects in human NSCLC lines. We also noticed that once mutant *Kras* was extinguished in *Cdkn2ab* null lung cancer cell lines, it could no longer be induced. This observation leads to the speculation that mutant *KRAS* facilitates the maintenance of a cell state that is permissive to the activity of the CC10 promoter driving the expression of rtTA or the accessibility of the Tet-op element driving mutant *KRAS*.

Overall, our data provide new genetic evidence that the loss of the three genes residing in *CDKN2AB* promote mutant *KRAS* lung tumorigenesis by fostering cellular

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proliferation, cancer cell differentiation, and metastatic behavior. We propose that mutant *Kras;Cdkn2ab* null mice provide a platform to accurately model aggressive mutant *Kras* lung adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Schuster, N. Venkateswaran, P.P. Scaglioni
Development of methodology: S. Peña-Llopis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Schuster, N. Venkateswaran, S. Peña-Llopis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Schuster, N. Venkateswaran, L. Girard, S. Peña-Llopis, P.P. Scaglioni
Writing, review, and/or revision of the manuscript: K. Schuster, N. Venkateswaran, A. Rabellino, L. Girard, S. Peña-Llopis, P.P. Scaglioni
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Schuster, N. Venkateswaran, A. Rabellino, S. Peña-Llopis
Study supervision: P.P. Scaglioni

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38. Konstantinidou G, Bey EA, Rabellino A, Schuster K, Maira MS, Gazdar AF, et al. Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring K-RAS mutations. Cancer Res 2009;69:7644–52.
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BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Scaglioni, Pier Paolo	Associate Professor
eRA COMMONS USER NAME (credential, e.g., agency login) scagliop	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Modena, Italy	M.D.	10/1989	Medicine
University of Modena, Italy	Resident	1989-1994	Internal Medicine
Fox Chase Cancer Center	Visiting Scientist	1991-1993	Virology
Massachusetts General Hospital	Research fellow	1994-1998	Virology
Montefiore Medical Center, Bronx, NY	Resident	1998-2001	Internal Medicine
Memorial Sloan Kettering Cancer Center, New York, NY	Clinical Fellow	2001-2005	Medical Oncology

A. Personal Statement

I am a medical oncologist and physician-scientist trained in molecular virology and cancer biology. I have built a productive independent research that has lead to peer reviewed publications and competitive funding (which includes several research fellowships for post-doctoral fellows). My laboratory is interested in understanding how genetic alterations contribute to tumorigenesis, modify response to cancer treatment or create vulnerabilities that may be targeted by therapeutics. To enable our research, we integrate the use of mouse cancer models, cell lines generated from human tumors, small molecule inhibitors and RNAi libraries. We have been working on mutant KRAS lung tumorigenesis and on acute promyelocytic leukemia because these cancer types depend on their initiating oncogenes. With our lung cancer research program, we have identified several vulnerabilities of mutant KRAS including a novel RHOA-FAK axis, which is essential for the maintainance of mutant KRAS adenocarcinoma. This work led to a clincial trial to investigate the effectivenes of FAK inhibitors in lung cancer (NCI identifier NCT01951690). In related work, we found mutant KRAS reprograms lipid homeostasis by regulating Acyl-CoA synthetase long-chain family member 3, establishing a reliance on fatty acids metabolism and β-oxidation. With our work on the promyelocytic tumor suppressor, we have found that the PIAs1 SUMO E3 ligase is a critical regulator of PML, PML-RARA and several oncogenic networks.

B. Positions and Honors

Positions and Employment

1989-1994 Resident in Internal Medicine, Department of Medicine, University of Modena Medical School, Italy. Program Director: B. Bonati MD.
1991-1993 Visiting Scientist, Department of Molecular Virology, Fox Chase Cancer Center, Philadelphia, PA. Research Advisor: C. Seeger, Ph.D.
1993-1998 Research Fellow in Medicine, Massachusetts General Hospital, Boston, MA. Research Advisor: J. Wands, MD.
1998-2001 Resident in Medicine, Montefiore Medical Center, The University Hospital for the Albert Einstein College of Medicine, Bronx, NY. Program Director: Joan Casey, MD.
2001- 2005: Fellow in Hematology/Oncology, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY. Program Directors: D. Bajorin, MD & M. Heaney, MD.
2002-2005: Post-Doctoral Fellow, Department of Pathology and Cancer Biology & Genetics Program, Sloan-Kettering Institute, New York, NY. Research Advisor: Pier Paolo Pandolfi, MD, Ph.D.

2005-2006. Instructor in Medicine, Memorial Sloan-Kettering Cancer, Department of Medicine, Division of Hematologic Oncology, Hematology Service, New York, NY.
2006-2014. Assistant Professor, Department of Medicine, Division of Hematology/Oncology, UT Southwestern Medical Center. Dallas, TX.
2014-Present. Associate Professor with Tenure, Department of Medicine, Division of Hematology/Oncology, UT Southwestern Medical Center. Dallas, TX.

Honors

1989 Medicina Prize for Italian Researchers younger than 35 years of age; 1991 Terme di Chianciano S.p.A. Fellowship; 2001 Montefiore Medical Center/A. Einstein College of Medicine**:** Citation in recognition as an outstanding House Officer; 2003 ASCO Young Investigator Award; 2003 CALGB Oncology Fellows Award 2003 ASH Travel Award; 2004 Michael and Ethel L. Cohen Fellow; 2004 Charles A. Dana Fellow, Clinical Scholars Training Program in Biomedical Research; 2004 Doris Duke Dinner Award
2004 ASH Travel Award; 2008 Gibbie Award Ryan Gibson Foundation
2012 Gibbie Award Ryan Gibson Foundation; 2014 Texas 4000 for Cancer Award.

Peer review Committees

2013-Present AACR Basic Cancer Research Fellowships Scientific Review Committee
2013-Present NCI Study Section J

Board Certifications

2001 American Board of Internal Medicine
2004 American Board of medical Oncology

C. Selected peer-reviewed publications (selected from 35 peer-reviewed publications)

Original research and theoretical treatises:

1. **P. P. Scaglioni***, M. Melegari*, and J. R. Wands. (*First authorship shared) Posttranscriptional regulation of hepatitis B virus replication by the precore protein. Journal of Virology. 1997. 71: 345-353. PMID: 8985356.
2. M. Melegari***, P. P. Scaglioni***, and J. R. Wands. (*First autorship shared) Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. Hepatology. 1998. 27: 628-33. PMID: 9462667
3. M. Melegari***, P. P. Scaglioni***, and J. R. Wands. (*First authorship shared) Cloning and characterization of a novel hepatitis B virus x binding protein that inhibits viral replication. Journal of Virology. 1998. 72: 1737-43. PMID: 9499022.
4. C. Gurrieri, P. Capodieci, R. Bernardi, **P.P. Scaglioni**, K. Nafa, L.J. Rush, D.A. Verbel, C. Cordon-Cardo, P.P. Pandolfi. J. Natl. Cancer Inst. 2004. 96:269-79. PMID: 14970276.
5. R. Bernardi, **P. P. Scaglioni**, S. Bergmann, H.F. Horn, K.H. Vousden and P. P. Pandolfi. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat. Cell. Biol. 2004. 6:665-672. PMID: 15195100.
6. H. Matsushita, **P.P. Scaglioni**, M. Bhaumik, E.M. Rego, L.F. Cai, Hayato Miyachi, A. Kakizuka, W.H. Miller, P.P. Pandolfi. In vivo analysis of the role of aberrant histone deacetylase recruitment and RARalpha blockade in the pathogenesis of acute promyelocytic leukemia. J Exp Med. 2006. 17:821-828. PMID: 16549595.
7. L.C. Trotman, A. Alimonti, **P.P. Scaglioni**, J.A. Koutcher, C. Cordon-Cardo, P.P. Pandolfi. Identification of a tumour suppressor network opposing nuclear Akt function. Nature. 2006. 25:523-527. PMID: 16680151.
8. **P.P Scaglioni**, T. Yung, L. F. Cai, H. Erdjument-Bromage, P. Tempst, J. Teruya-Feldstein, P. P. Pandolfi. A CK2-dependent pathway for PML degradation upon cellular and oncogenic stress. Cell. 2006. 126:269-83. PMID: 16873060.
9. T.H. Shen, H.K. Lin, **P.P. Scaglioni**, T.M. Yung, P.P. Pandolfi. The Mechanisms of PML-Nuclear Body Formation. Mol Cell. 2006. 24:331-9. PMID: 17081985.
10. **P.P. Scaglioni**, T.M. Yung, S.C. Choi, C. Baldini, G. Konstantinidou and P.P. Pandolfi. CK2 mediates phosphorylation and ubiquitin mediated degradation of the PML tumor suppressor. Mol. Cell. Biochem. 2008. 316:149-54. PMID: 18566754.
11. G. Konstantinidou, E.A. Bey, A. Rabellino, K. Schuster, M.S. Maira, A.F. Gazdar, D. Boothman, **P.P. Scaglioni**. Dual PI3K/mTOR blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer. Cancer Research. 2009. 69:7644-52. PMID: 19789349.

12. J.P. Sullivan, M. Spinola, M. Dodge, M.G. Raso, C. Behrens, B. Gao, K. Schuster, C. Shao, J.E. Larsen, L.A. Sullivan, S. Honorio, Y. Xie, **P.P. Scaglioni**, J.M. DiMaio, A.F. Gazdar, J.W. Shay, I.I. Wistuba, J.D. Minna. Aldehyde dehydrogenase activity selects for Notch signaling dependent non-small cell lung cancer stem cells. Cancer Research. 2010. 70:9937-48. PMID: 21118965.
13. H.V. Naina, D. Levitt, M. Vusirikala, L.D. Anderson Jr, **P.P. Scaglioni**, A. Kirk, R.H. Collins Jr. Successful treatment of relapsed and refractory extramedullary acute promyelocytic leukemia with tamibarotene. J Clin Oncol. 2011. 29:e534-6. PMID: 21482998.
14. **P.P. Scaglioni**, L. F. Cai, S.M. Majid, T.M. Yung, N.D. Socci, S.C. Kogan, L. Kopelovich, P.P. Pandolfi. Treatment with 5-Azacytidine Accelerates Acute Promyelocytic Leukemia Leukemogenesis in a Transgenic Mouse Model. Genes Cancer. 2011. 2: 160–165. PMCID: PMC3111249.
15. K. Schuster, J. Zheng, A.A. Arbini, C.C. Zhang, **P.P. Scaglioni**. Selective targeting of the mTORC1/2 protein complexes leads to antileukemic effects in vitro and in vivo. Blood Cancer Journal. 2011. 1, e34. doi:10.1038/bcj.2011.30. PMID: 22829195.
16. **P.P. Scaglioni***, A. Rabellino, T.M. Yung, R. Bernardi, S. Choi, G. Konstantinidou, C. Nardella, K. Cheng, P.P. Pandolfi*. ***Co-corresponding authors**. Translational-dependent mechanisms lead to PML upregulation and mediate oncogenic K-RAS induced cellular senescence. EMBO Molecular Medicine. 2012. 4:594-602. PMID: 22359342.
17. A. Rabellino, B. Carter, G. Konstantinidou, S.Y. Wu, A. Rimessi, L. A. Byers, J.V. Heymach, Luc Girard, C.M Chiang, J. Teruya-Feldstein and **P. P. Scaglioni**. The SUMO E3-ligase PIAS1 regulates the tumor suppressor PML and its oncogenic counterpart PML-RARA. Cancer Research, 2012. 72:2275-84. PMID: 22406621.
18. G. Konstantinidou, A. Heguy, F. Torti, G. Ramadori, K. Kangasniemi, R.E. Ramirez, Y. Cai, C. Behrens, M.T. Dellinger, I.I. Wistuba, R.A. Brekken, J. Teruya-Feldstein and **P.P. Scaglioni**. RHOA-FAK signaling is required for the maintenance of K-RAS-driven adenocarcinomas. Cancer Discovery, 2013. 3:444-57. Article **featured in World Biomedical Frontiers**.
19. K. Schuster, N. Venkateswaran, A. Rabellino, L. Girard, S. Peña-Llopis and **P.P. Scaglioni**. Loss of the *CDKN2AB* locus promotes mutant *KRAS* lung tumorigenesis. Molecular Cancer Research, 2014 Mar 11. [Epub ahead of print]. PMID: 24618618.

Non-experimental articles, books and chapters:

1. **P.P. Scaglioni** and P.P. Pandolfi. Taking apart a cancer protein. Nature. 2003. 426: 512-13. PMID: 14654828.
2. A. Rabellino and **P.P. Scaglioni**. PML degradation: multiple ways to eliminate PML. Front. Oncol. PMID: 2013:3:60. PMID: 23526763.
3. A. Rabellino and **P.P. Scaglioni.** PIAS1 (protein inhibitor of activated STAT, 1). Atlas Genet Cytogenet Oncol Haematol. July 2013. URL : http://AtlasGeneticsOncology.org/Genes/PIAS1ID45688ch15q23.html.

D. Research Support

Ongoing Research Support

CPRIT RP140672 8/31/2014-8/30/2017
Individual Investigator Research Award
Mutant KRAS reprograms lipid metabolism exposing beta-oxidation as a novel therapeutic target in lung cancer lung cancer.
Major goal: with this grant we propose to determine the role of fatty acid beta-oxidation in metabolic reprogramming of mutant KRA sung cancer cells.
Role: PI

Texas 4000 One time gift 2014
Exploitation of Fatty Acid β-oxidation as a novel therapeutic target in lung cancer
This research grant will test whether inhibition of fatty acid beta-oxidation is a novel therapeutic strategy in lung cancer.
Role: PI

RO1 CA 137195A1 7/1/2009-6/30/2015 (no cost extension)
NIH/NCI
Characterization and Drug Targeting of the PML Tumor Suppressor in Lung Cancer.
Major Goal: this grant proposes studies to identify the mechanisms that mediate PML tumor suppressive function with an approach that integrates a biochemical and molecular approach with studies in mouse models of lung cancer.
Role: PI

Grant # LC110229 9/1/2012-8/31/2015
CDMRP LCRP Investigator-Initiated Research Award
Deconstruction of oncogenic K-RAS signaling reveals focal adhesion kinase as a novel therapeutic target in NSCLC
Major Goal: with this grant we will characterize the role of FAK signaling in mutant K-RAS induced NSCLC tumorigenesis
Role: PI

Grant #13-068-01-TBG 1/1/2013-12/31/2016
American Cancer Society Scholar Award
Identification of critical components of the K-RAS network in lung cancer
Major Goal: with this grant we will identify vulnerabilities of mutant K-RAS lung cancer.
Role: PI

Grant #RP101251
Cancer Prevention and Research Institute of Texas (CPRIT) 9/1/2012-8/31/2017
Development of Nuclear Receptor and Coregulator Profiles for Diagnostic and Therapeutic for diagnostic and therapeutic (theragnostic) targeting of breast and lung cancers.
Role: collaborator
Major goal: this grant proposes to determine whether nuclear receptors and nuclear co-receptors can be used as risk stratifiers or therapeutic targets in lung and breast cancers.

5P50 CA70907-15 (Minna) 9/1/98-4/30/19 (NCE)
NIH/NCI
University of Texas SPORE (Special Program of Research Excellence) in Lung Cancer
The overall goal is the translation of findings to and from the laboratory and the clinic to result in improvement in the diagnosis, treatment, and prevention of lung cancer. It is a joint effort of UTSW and MDACC.
Role: Co-PI of Project #3 "Preclinical Development and Clinical Testing of MEK and PI3K Targeted Therapy for KRAS-mutant NSCLC as a Method of Radiosensitization and Metastasis Inhibition"

Completed Research Support

The Ryan Gibson Foundation 6//1/2012-5/31/2013
Private Foundation
Role of the PIAS1 E3 SUMO ligase in APL
Major Goal: this grant proposes to characterize the role of SUMOylation in promoting APL leukemogenesis and response to arsenic trioxide treatment.
Role: PI

K08 CA 112325-01 (Scaglioni) 8/1/2005-7/31/2010
NIH/NCI
Post-transcriptional regulation of PML function

Major Goal: this career development award focused on the characterization of the mechanisms underlying aberrant PML protein degradation in cancer cells and on the mechanisms controlling PML tumor suppressive functions both in vitro and in vivo.
Role: PI

Concern Foundation Conquer Cancer Now (Scaglioni) 7/1/2008-6/30/2010
Private Foundation
Exploiting oncogene addiction in lung cancer therapy
Major Goal: the goals of this grant were to: 1. Characterize of the mechanisms that mediate PML protein tumor suppressive function and to study the effects of PML pharmacologic upregulation; 2. Characterize the effects of role of the PI3K pathway in the pathogenesis of non-small cell lung cancer in the mouse.
Role: PI

Leukemia Texas (Scaglioni) 8/1/2008-12/31/2010
Private Foundation
Development of a novel targeted therapy for acute and chronic leukemias
Major Goal: this grant explored the functional significance of the PI3K pathway in the pathogenesis of chronic myelogenous leukemia using hematopoietic stem cells and mouse models of leukemia.
Role: PI